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(54) **Homogeneous recombinant immune interferon fragments and pharmaceutical compositions containing same.**

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EP 0 256 424 B1

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## Description

The present invention relates to homogeneous recombinant human immune interferon fragments having 6 to 11 amino acids deleted at the carboxy terminus as compared to full-length mature recombinant human immune interferon (amino acid sequence shown in Figure 1). The invention also relates to replicable microbial expression vehicles comprising a nucleotide sequence encoding a recombinant immune interferon fragment defined above, to microorganisms transformed with these expression vehicles and to processes for the preparation of said interferon fragments and said microorganisms. The invention further relates to pharmaceutical compositions containing one or more of these recombinant immune interferon fragments and to the use of these recombinant immune interferon fragments and pharmaceutical compositions for the treatment of various disease states.

Natural human immune interferon (IFN- $\gamma$ ) is produced upon mitogenic stimulation of lymphocytes. It exhibits antiviral and anti-proliferative activity. This activity is pH 2 labile. The functional form of the immune interferon may be a multimer, most likely a dimer, a trimer or a tetramer (Pestka et al., J. Biol. Chem. 258, 9706-9709 [1983]).

It has been found that immune interferon is encoded in the human genome by a single gene which codes for a precursor-polypeptide of 166 amino acid residues (Derynck et al., Nucleic Acids Res. 10, 3605-3615 [1982]). Post-translational processing was believed to result in a polypeptide comprising 146 amino acids and having a Cys-Tyr-Cys-Gln... N-terminal sequence.

Using methods of the recombinant DNA technology (see for example Maniatis et al., "Molecular cloning - A laboratory manual", Cold Spring Harbor Laboratory, 1982) expression vectors coding for a recombinant immune interferon comprising these 146 amino acids have been constructed. Upon introduction of these expression vectors into a microbial host, recombinant immune interferon polypeptides consisting of 147 amino acids, namely the 146 amino acids mentioned above and an additional N-terminal methionine, are synthesized. The additional methionine derives from the mRNA translational start signal AUG which codes for the amino acid methionine. In the parental human gene this translational start signal is situated in front of the signal peptide. This signal peptide is cleaved off upon post-translational processing, together with the methionine derived from the translational start signal leading to methionine-free mature immune interferon consisting of 146 amino acids. Prokaryotic hosts are not able to cleave the signal peptide in front of the human immune interferon precursor polypeptide at the right position. Therefore the coding sequence for the signal peptide in the immune interferon gene has to be removed by genetic engineering in order to express the immune interferon in mature form. The ATG codon directly in front of the coding sequence for the mature polypeptide results in the N-terminal methionine present in recombinant human immune interferon. In spite of the additional N-terminal methionine and despite the fact that it is not glycosylated the recombinant immune interferon exhibits antiviral and anti-proliferative activity coupled with pH 2 lability comparable to the immune interferon isolated from human lymphocytes (Gray et al., Nature 295, 503-508 [1982]). Later on Rinderknecht et al., (J. Biol. Chem. 259, 6790-6797 [1984]) have shown that immune interferon purified from induced human peripheral blood lymphocytes consists of a polypeptide having 143 amino acid residues, lacking the Cys-Tyr-Cys- sequence and having a pyroglutamate residue at its N-terminus. Some heterogeneity at the carboxy terminus was observed. Reengineering of the original recombinant immune interferon gene led to expression vectors coding for mature recombinant immune interferon comprising these 143 amino acids and having as outlined above an additional methionine at the N-terminus. Comparison of the biological activities of the recombinant human immune interferons with or without the Cys-Tyr-Cys- sequence showed, that the deletion of the Cys-Tyr-Cys- sequence led to a two-fold higher antiviral activity. (European Patent Application No. 146 354, published on 26.6.85). Moreover it is shown in European Patent Application No. 146 354 that immune interferon fragments generated by limited tryptic digestion of mature recombinant human immune interferon, had decreased antiviral activity, compared to a recombinant immune interferon preparation consisting of a mixture of interferon polypeptides (= reference material) having 139 or 143 amino acids (ratio 98:2). For example a fragment consisting of 131 amino acids, lacking 12 C-terminal amino acids, showed 40-50% specific activity compared to the above mentioned reference material. Fragments with additional 3 or 6 amino acids removed from the C-terminus had 6-9 or 1% respectively of the specific activity of the reference material. Therefore in view of the reduced activity exhibited by the immune interferon fragments exemplified in the above mentioned European Patent Application, it can be concluded that all fragments lacking terminal amino acids will have a lower antiviral activity than the above-mentioned reference material or the mature recombinant human immune interferon with 143 amino acids lacking the Cys-Tyr-Cys- sequence at the N-terminus. On the other hand these immune interferon fragments have been generated only by limited tryptic digestion. Trypsin catalyzes the hydrolysis of peptide bonds, whose carbonyl function is donated by a basic amino acid,

usually arginine or lysine. There are 20 lysine residues and 8 arginine residues in the amino acid sequence of immune interferon. The amount of the individual fragments generated by trypsin depends on the accessibility of the peptide bond which has to be cut to generate the fragment. Nevertheless, limited proteolytic digestion leads to a wide spectrum of fragments and the purification of a specific fragment to homogeneity from such a mixture is highly problematic in particular since polypeptides having essentially the same amino acid sequence but differing in size by a few amino acids would have to be separated. Under the prevailing conditions the fractionation by HPLC described in European Patent Application No. 146 354 is not considered as adequate for purifying the immune interferon fragments exemplified therein to homogeneity, that means that they are obtained substantially free from other immune interferon fragments differing in size. Moreover, due to the specificity of trypsin, the immune interferon fragments of the present invention having non-basic C-terminal amino acids like serine, glutamine, methionine, leucine or phenylalanine cannot be generated by tryptic digestion. This may be the reason why only immune interferon fragments with 125, 129, 131 and 139 amino acids, having either lysine or arginine as C-terminal amino acid are exemplified in European Patent Application No. 146 354. Although a method for the preparation of immune interferon fragments by recombinant DNA technology is generally described in the above mentioned European Patent Application, recombinant human immune interferon fragments comprising 132 to 137 amino acids counted from the first glutamine residue in the amino acid sequence shown in Figure 1, have been prepared for the first time in homogeneous form by the methods of the present invention. Most surprisingly it has been found that these homogeneous recombinant immune fragments have an increased specific antiviral activity compared to full length mature recombinant immune interferon. Similarly it was observed that the other biological activities exhibited by immune interferon (reviewed by Trinchieri et al., Immunology Today 6, 131-136 [1985]) are also increased for the recombinant immune interferon fragments of the present invention in comparison to the mature recombinant immune interferon. Pharmaceutical compositions containing one or more of the recombinant immune interferon fragments of the present invention can be used for the treatment of various disease states. Examples of such disease states are: viral infections, neoplastic diseases or rheumatoid arthritis.

The present invention therefore provides homogeneous recombinant immune interferon fragments exhibiting a specific antiviral activity higher than mature recombinant immune interferon and having the amino acid sequence

X-Y-Asp-Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Ala-Gly-His-Ser-Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-Gly-Ile-Leu-Lys-Asn-Trp-Lys-Glu-Glu-Ser-Asp-Arg-Lys-Ile-Met-Gln-Ser-Gln-Ile-Val-Ser-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-Lys-Asp-Asp-Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-Asp-Met-Asn-Val-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-Lys-Leu-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-Gln-Arg-Lys-Glu-Ile-His-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-Ser-Pro-Ala-Ala-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Z

wherein either X is a methionine and Y is a glutamine residue or X is hydrogen and Y is a glutamine or a pyroglutamate residue, and Z is

Ser,  
Ser-Gln,  
Ser-Gln-Ile,  
Ser-Gln-Met-Leu,  
Ser-Gln-Met-Leu-Phe or  
Ser-Gln-Ile-Leu-Phe-Arg.

These homogeneous recombinant immune interferon fragments have a significant higher biological activity compared to mature recombinant human immune interferon, that means to a full-length recombinant immune interferon consisting of 143 amino acids, having either a glutamine or a pyroglutamate residue at position 1 of its amino acid sequence. When the amino acid residue at position 1 is glutamine, the full-length recombinant immune interferon may contain optionally an additional methionine residue as 144th amino acid at its N-terminus. The additional methionine residue derives from the mRNA translational start signal AUG which codes for the amino acid methionine as outlined above. In expression systems like E.coli this methionine is not always processed away. The additional N-terminal methionine has been found not to impair the biological activity of most recombinant polypeptides (Winnacker, in "Gene und Klon", p. 255, VCH, Weinheim, Germany [1985]). Upon removal of the N-terminal methionine in recombinant immune interferon fragments the newly generated N-terminal glutamine residue may cyclize to the pyroglutamate form, again without any believed impairment of the biological activity. The recombinant

immune interferon fragments of the present invention may be in multimerized form, preferably they are present as dimers, trimers or tetramers.

The recombinant immune interferon fragments of the present invention can be used to prepare pharmaceutical compositions consisting of a significantly reduced amount of said recombinant immune interferon fragments as compared to the amount of mature recombinant human immune interferon needed, to obtain essentially the same antiviral activity and a physiological compatible carrier. Such pharmaceutical compositions are useful for the treatment of various disease states. It is understood that amino acid substitutions, particularly single amino acid substitutions in the recombinant immune interferon fragments disclosed herein, giving rise to variants of the recombinant immune interferon fragments having higher specific activity compared to mature recombinant human immune interferon are possible. It is within the state of the art to introduce such substitutions in a recombinant polypeptide. More generally the present invention includes all recombinant immune interferon fragments having 6 to 11 amino acids deleted at the C-terminus as compared to a full-length mature recombinant human immune interferon or modifications and allelic variations thereof, which fragments exhibit biological activity greater than the full-length mature recombinant human immune interferon. The invention also provides replicable microbial expression vehicles comprising a nucleotide sequence encoding a recombinant immune interferon fragment operably linked to an expression control sequence and microorganisms transformed with such an expression vehicle. Moreover it provides pharmaceutical compositions containing one or more recombinant immune interferon fragments and a physiological compatible carrier. The invention further relates to processes for the preparation of recombinant immune interferon fragments through recombinant DNA technology, to processes for the preparation of a microorganism transformed with a replicable microbial expression vehicle mentioned above and to methods for the preparation of pharmaceutical compositions containing such recombinant immune interferon fragments. Furthermore the invention relates to the use of these interferon fragments and corresponding pharmaceutical compositions for the treatment of various disease states.

The present invention may be more readily understood by reference to the following detailed description of the invention when considered in connection with the accompanying drawings, wherein the following abbreviations and symbols are used:

B, E, H, S, Sa, Sc, X, Xb which indicate sites for restriction endonucleases BamHI, EcoRI, HindIII, SphI, SalI, ScaI, XhoI and XbaI, respectively.

In figures 2, 4 and 6 to 9



represents promoters of the genes bla, lacI and neo;



represents ribosomal binding sites of the genes bla, cat, neo and lacI;



represents terminators  $t_0$  and T1;



represents the regulatable promoter/operator element  $P_{N25X/O}$ ;



represents ribosomal binding site RBSII, SphI;  $\rightarrow$  represents coding regions under control of this ribosomal binding site;  $=$  represents regions required for DNA replication (repl.);



represents coding regions for dihydrofolate reductase (dhfr), chloramphenicol acetyltransferase (cat),  $\beta$ -lactamase (bla), lac repressor (lacI), neomycin phosphotransferase (neo), and interferon- $\gamma$  (IFN- $\gamma$ ).

Figure 1

Amino acid sequence of mature recombinant human immune interferon (rIFN- $\gamma$ ) lacking the Cys-Tyr-Cys- sequence at the N-terminus and the nucleotide sequence encoding it. The HinI site used for the construction of the recombinant immune interferon fragments is underlined. The N-terminal methionine derived from the prokaryotic translational start signal is put in parenthesis.

Figure 2

Schematic drawing of the plasmid pDS8/RBSII,SphI.

Figure 3

Nucleotide sequence of the XhoI/XbaI fragment of the plasmid pDS8/RBSII,SphI containing the regulatable promoter/operator element  $P_{N25X10}$ , the ribosomal binding site RBSII,SphI, the dhfr-gene, terminator t<sub>0</sub>, the cat-gene and terminator T<sub>1</sub>. The restriction endonuclease sites indicated in Fig. 2 are overlined and the region under control of RBSII,SphI encoding a dihydrofolate reductase polypeptide is underlined. In addition the pBR322 entity of pDS8/RBSII,SphI is schematically shown, where the given numbers refer to the nucleotide sequence of pBR322 (J.G. Sutcliffe, Cold Spring Harbor, Symp. Quant. Biol. 43, pp. 77-90 [1979]).

Figure 4

Schematic drawing of the plasmid pDMI,1.

Figure 5

DNA sequence of the plasmid pDMI,1. The restriction endonuclease sites indicated in Fig. 4 are overlined and the coding regions for neomycin phosphotransferase (neo) and lac repressor (lacI) are underlined.

Figure 6

Schematic description of the construction and isolation of fragment 1 containing the gene for mature recombinant human immune interferon (rIFN- $\gamma$ ) starting from plasmid pRC23/IFI-900 containing the gene coding for a recombinant human interferon consisting of 146 amino acids, having Cys-Tyr-Cys- as N-terminal sequence.

Figure 7

Integration of fragment 1 into pDS8/RBSII,SphI, resulting in pGLS. (Sc) in the schematic drawing of pGLS indicates the position where fragment 1 is linked to the ScI cleavage site of pDS8/RBSII,SphI.

Figure 8

Schematic description of the construction of the EcoRI-HindIII-fragments F(-6), F(-8) and F(-11) coding for the C-terminus of three different recombinant immune interferon fragments using a HinI-fragment of pGLS and the HindIII-HindIII-adapters A(-6), A(-8) and A(-11). The EcoRI-HindIII-fragments F(-7), F(-9) and F(-10) were constructed in similar way using the HinI-HindIII-adapters

A(-7)      AGTCAGATGCTGTTTTAA  
                  GTCTACGACAAAATTTCTGA  
 5            A(-9)      AGTCAGATGTAA  
                  GTCTACATTTCTGA  
                  A(-10)    AGTCAGTAA  
                  GTCATTTCTGA

In the sequence given the relevant codons for translational termination are underlined.

#### Figure 9

Schematic description of the integration of fragments F(-6), F(-8) and F(-11) into the EcoRI- and HindIII-cleavage sites of pDS8/RBSII, SphI resulting in plasmids pIFN- $\gamma$ (-6), pIFN- $\gamma$ (-8) and pIFN- $\gamma$ (-11). The fragments F(-7), F(-9) and F(-10) were integrated in similar way resulting in plasmids pIFN- $\gamma$ (-7), pIFN- $\gamma$ (-9) and pIFN- $\gamma$ (-10).

#### Figure 10

Amino acid sequences of the gene products rIFN- $\gamma$ , IFN- $\gamma$ (-6), IFN- $\gamma$ (-7), IFN- $\gamma$ (-8), IFN- $\gamma$ (-9), IFN- $\gamma$ (-10) and IFN- $\gamma$ (-11) which are obtained upon expression of pGLS, pIFN- $\gamma$ (-6), pIFN- $\gamma$ (-7), pIFN- $\gamma$ (-8), pIFN- $\gamma$ (-9), pIFN- $\gamma$ (-10) and pIFN- $\gamma$ (-11), respectively. (130aa) means the 130 amino acids from position 1 to position 130 (see Fig. 1)

#### Figure 11

Analysis of the mature human immune interferon and the human interferon fragments by SDS-polyacrylamide gelelectrophoresis. In lanes a, b, c and d (Fig. 11a, part A) and lanes 1 to 9 (Fig. 11b) lysates of E. coli M15 cells, harbouring pDMI,1 and in addition the expression vector indicated below, are shown:

| <u>expression vector</u> | <u>Figure 11a</u> | <u>Figure 11b</u> |
|--------------------------|-------------------|-------------------|
|                          | lane              | lane              |
| pGLS                     | a                 | 1, 9              |
| pIFN- $\gamma$ (-4)      | -                 | 2                 |
| pIFN- $\gamma$ (-5)      | -                 | 3                 |
| pIFN- $\gamma$ (-6)      | b                 | 4                 |
| pIFN- $\gamma$ (-8)      | c                 | 5                 |
| pIFN- $\gamma$ (-9)      | -                 | 6                 |
| pIFN- $\gamma$ (-10)     | -                 | 7                 |
| pIFN- $\gamma$ (-11)     | d                 | 8                 |

In lanes a, b, c and d (Fig. 11a, part B) and lanes 10 to 17 and lane 20 (Fig. 11b) the purified interferon polypeptides isolated from the lysates a, b, c and d (Fig. 11a, part A) and lanes 1 to 9 (Fig. 11b) respectively are shown. The human immune interferon fragments IFN- $\gamma$ (-14) and IFN- $\gamma$ (-18) prepared by limited proteolysis are shown in lanes 18 and 19 respectively.

MW (Fig. 11a) and M (Fig. 11b) denote a protein size marker (BIO-RAD laboratories) consisting of proteins of 14.4 kd, 21.5 kd, 31 kd, 45 kd, 66.2 kd and 92.5 kd molecular weight (1 kd = 1'000 dalton).

#### Figure 12

5

Antiviral activity in relation to the carboxyterminal sequence of IFN- $\gamma$ . The carboxyterminal sequence is given in the one letter code described by Dayhoff et al., ("Atlas of Protein Sequence and Structure", M.O. Dayhoff, ed., Vol. 5, p. 17, Natl. Biomed. Res. Found., Silver Spring, Maryland, U.S.A., [1979]) on the abscissa. The antiviral activity exhibited by the recombinant immune interferon fragments identified on the abscissa by the number of amino acids deleted at the carboxyterminus in comparison to the full-length mature recombinant human immune interferon (identified by "O") are plotted on the ordinate. Each point represents the geometric mean titer of at least six independent determinations. Bars indicate the corresponding 95 % confidence limits.

#### Figure 13

15

Comparison of the antiviral activity, the macrophage activation and the receptor binding capacity in relation to the carboxyterminal sequence of IFN- $\gamma$  (for details see legend to Fig. 12 and text). The values for the antiviral activity, the macrophage activation and the receptor binding capacity of the immune interferon fragments in relationship to the corresponding values of mature recombinant immune interferon, which were arbitrarily taken as 1, are shown on a logarithmic scale. The absolute value for the macrophage activation of mature recombinant immune interferon was  $5 \times 10^5$  U/mg.

In a preferred embodiment of the present invention recombinant immune interferon fragments can be prepared by selection of a suitable plasmid containing a full-length copy of the human immune interferon gene or allelic variants thereof. Translational stop codons can then be introduced in the region of the gene coding for the C-terminus of immune interferon either by site directed mutagenesis as described by Smith et al. (in "Genetic Engineering" 3, 1-32 [1981], J.K. Setlow, A. Hollaender eds., Plenum Press, New York) or by removing a suitable restriction fragment from this region and by replacing this restriction fragment with a synthetic DNA fragment coding for the desired C-terminus of the recombinant immune interferon fragment. In the latter method preferably two different restriction endonucleases are used to cut the starting plasmid, generating two different sticky ends. The synthetic DNA containing complementary sticky ends can then be integrated directly in the right orientation. In a next step the DNA sequence coding for the recombinant immune interferon fragment can be inserted in a replicable microbial expression vehicle, comprising an origin of replication, a promoter or promoter-operator and a sequence encoding a ribosomal binding site (RBS). Suitable replicable expression vehicles can be found in Maniatis et al. (supra) or in the examples of the present invention. Preferred replicable expression vehicles comprising a nucleotide sequence encoding a recombinant immune interferon fragment operably linked to an expression control sequence are pIFN- $\gamma$ (-6), pIFN- $\gamma$ (-7), pIFN- $\gamma$ (-8), pIFN- $\gamma$ (-9), pIFN- $\gamma$ (-10) and pIFN- $\gamma$ (-11), wherein the coding sequence of the recombinant immune interferon fragments are integrated in the expression vector pDS8/RBSII, SphI (Fig. 2). The expression vector pDS8/RBSII, SphI was introduced into E.coli M15 (pLE-11) a strain of E.coli (E.coli DZ291) transformed with plasmid pDMI,1 (Fig. 4) which plasmid encodes the lac repressor. The resulting E.coli M15 (pDS8/RBSII, SphI; pDMI,1) was deposited on August 6, 1989 at the Deutsche Sammlung von Mikroorganismen (DSMZ) accession number being DSM 3809. The methods for the construction of the expression vehicles coding for the recombinant immune interferon fragments IFN- $\gamma$ (-6), pIFN- $\gamma$ (-7), IFN- $\gamma$ (-8), IFN- $\gamma$ (-9), IFN- $\gamma$ (-10), and IFN- $\gamma$ (-11) are outlined in detail in the examples. Based on these examples it is within the skill of an artisan in the field to construct expression vehicles capable of expressing the recombinant immune interferon fragments of the present invention. The replicable expression vehicles capable of expressing recombinant immune interferon fragments are then transformed into a suitable host organism by the procedure described in the examples or as described by Maniatis et al. (supra). Depending on the host cell used the recombinant immune interferon fragments may be in glycosylated form or not. Preferred host organisms include *Escherichia coli* (e.g. strain M15 or strain W3110 [ATCC No. 27325]), *Bacillus subtilis* and so forth. The most preferred host organism of this invention is E.coli strain M15 mentioned above.

Once the organism capable of carrying out the expression of the polypeptides of the present invention have been prepared, the process of the invention can be carried out in a variety of ways, depending upon the nature of the construction of the expression vectors and upon the growth characteristics of the host. Typically, the host organism will be grown under conditions which are favorable for the production of large quantities of cells. After a large number of cells has accumulated, suitable inducers or derepressors in the

growth medium or a temperature-shift cause the control sequence supplied with such DNA sequence to become active, permitting the transcription and translation of the coding sequence. In the present invention the expression of the gene coding for the recombinant immune interferon fragment is inhibited by the lac repressor. When a large number of cells have accumulated, the interferon gene is expressed by the addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). The protein produced by the recombinant cell can be released by lysing the cell by conventional means well known in the art. The particular means of lysing will depend upon the host cell utilized. In the present invention lysing the cells with guanidium-hydrochloride is preferred.

The homogeneous recombinant immune interferon fragments of the present invention can be purified by any known method, such as by precipitation with ammonium sulfate, dialysis to remove salts (under normal or vacuum pressure), gel filtration, chromatography, preparative flat-bed iso-electric focusing, gel electrophoresis, high performance liquid chromatography, ion exchange chromatography, gel filtration and reverse phase chromatography, and affinity chromatography and the like, or by using monoclonal or polyclonal antibodies. The homogeneous recombinant immune interferon fragments obtained are essentially free of other proteins as can be judged by SDS-polyacrylamide gel electrophoresis or HPLC analysis.

The recombinant immune interferon fragments may form dimers, trimers or tetramers, that means that the purified recombinant immune interferon fragments may be present as a combination of two, three or four such fragments. The nature of the combining mechanism is unclear.

Antiviral activities of the recombinant immune interferon fragments IFN- $\gamma$ (-6), IFN- $\gamma$ (-7), IFN- $\gamma$ (-8), IFN- $\gamma$ (-9), IFN- $\gamma$ (-10), and IFN- $\gamma$ (-11) were determined in a bioassay based on reduction of the cytopathic effect as described by Rubinstein et al. (J. Virol. 37, 755-758 [1981]). Human amnion cells (FL-cells) and Sindbis virus as a challenge virus were used. Samples were prediluted to about 1000 units/ml and titrated on a microtiter plate in duplicate. A full-length mature recombinant immune interferon [sequence in Fig. 1] preparation (lot No. 85.20 SX-Pool) was used as working standard. It was calibrated to the NIH Reference Human Interferon Gamma Standard, No. Gg 23-901-530. The NIH-Standard has a titer of  $3.68 \pm 0.34 \log_{10}$  units/ampoule (n = 39).

The results are summarized in the following Table 1. Each value represents the mean of at least 13 separate determinations. Geometric mean titers were statistically evaluated in a t-test. Differences between the mature recombinant immune interferon and the recombinant immune interferon fragments are highly significant (P = 99%). The difference between the titers of IFN- $\gamma$ (-6), and IFN- $\gamma$ (-11) are also highly significant. The values of IFN- $\gamma$ (-6) and of IFN- $\gamma$ (-8) differ significantly (P = 95%). The potencies of IFN- $\gamma$ (-8) and IFN- $\gamma$ (-11), however, are not significantly different.

Table 1

Specific antiviral activity

|  |                       |
|--|-----------------------|
| Mature recombinant immune interferon (rIFN- $\gamma$ )     | $9.5 \cdot 10^6$ U/mg |
| Recombinant immune interferon fragment IFN- $\gamma$ (-6)  | $4.1 \cdot 10^7$ U/mg |
| Recombinant immune interferon fragment IFN- $\gamma$ (-8)  | $5.9 \cdot 10^7$ U/mg |
| Recombinant immune interferon fragment IFN- $\gamma$ (-11) | $7.0 \cdot 10^7$ U/mg |

In order to study the increased antiviral activity in more detail, further recombinant immune interferon fragments were constructed either as described above, e.g. IFN- $\gamma$ (-4) and IFN- $\gamma$ (-5), or by limited



proteolysis of full-length mature recombinant immune interferon, e.g. IFN- $\gamma$ (-1) was prepared using mouse submaxillary gland "Arg C" protease, which cleaves proteins specifically at the carboxylic side of arginine (Schenkein et al., *Arch. Biochem. Biophys.* 182, 64-70 [1977]), whereas IFN- $\gamma$ (-8) was prepared using the fibrinolytic enzyme plasmin.

- 5 The specific antiviral activity of these polypeptides was determined as described above. Table 2 and Figure 12 show that the recombinant immune interferon fragments IFN- $\gamma$ (-8), IFN- $\gamma$ (-9), IFN- $\gamma$ (-10) and IFN- $\gamma$ (-11) exhibit a remarkably higher specific antiviral activity than mature recombinant immune interferon or the immune interferon fragments IFN- $\gamma$ (-14) and IFN- $\gamma$ (-18) prepared by limited proteolysis.

Table 2

Specific antiviral activity (U/mg)  
(in brackets 95% confidence limits)

|    |                     |                    |   |
|----|---------------------|--------------------|---|
| 15 | rIFN- $\gamma$      | $1.91 \times 10^7$ | $(1.36 \times 10^7 - 2.47 \times 10^7)$ |
|    | IFN- $\gamma$ (-4)  | $2.88 \times 10^7$ | $(2.19 \times 10^7 - 3.60 \times 10^7)$ |
| 20 | IFN- $\gamma$ (-5)  | $4.79 \times 10^7$ | $(3.54 \times 10^7 - 6.47 \times 10^7)$ |
|    | IFN- $\gamma$ (-6)  | $3.39 \times 10^7$ | $(2.46 \times 10^7 - 4.66 \times 10^7)$ |
|    | IFN- $\gamma$ (-8)  | $7.08 \times 10^7$ | $(5.06 \times 10^7 - 9.91 \times 10^7)$ |
| 25 | IFN- $\gamma$ (-9)  | $9.33 \times 10^7$ | $(6.60 \times 10^7 - 1.32 \times 10^8)$ |
|    | IFN- $\gamma$ (-10) | $8.32 \times 10^7$ | $(6.72 \times 10^7 - 1.03 \times 10^8)$ |
|    | IFN- $\gamma$ (-11) | $6.31 \times 10^7$ | $(4.99 \times 10^7 - 7.97 \times 10^7)$ |

- 30 Similarly the immune interferon fragments IFN- $\gamma$ (-8), IFN- $\gamma$ (-9), IFN- $\gamma$ (-10) and IFN- $\gamma$ (-11) exhibited a remarkably higher antiproliferative activity and increased immunoregulatory activities.

- The antiproliferative activity can be determined by measuring the inhibitory action of the different immune interferons on the incorporation of radioactively labeled thymidine into cellular DNA. Three malignant cell lines (U 937, BS 14, LLC 1) and one normal fibroblast line (AG 152, passage no. 6-10) were used. U 937 is a myelomonocytic leukemia cell line, BS 14 was derived from a patient with malignant melanoma and LLC 1 from a patient with lung cancer. The cells were plated in 96 microwell plates in RPMI 1640 supplemented with 10% fetal calf serum, 1% L-glutamin (200 mM), 1% penicillin (100 u/ml) - streptomycin (100 ug/ml), 1% non-essential amino acids (100 x) and 1% sodium pyruvate (100 mM) at different densities according to their individual growth curves: 20'000 cells/ml for U 937 and AG 1523, 1'000 cells/ml for BS 14 and LLC 1. The cells were allowed to adhere for 24 hours. Mature recombinant immune interferon (rIFN- $\gamma$ ) and immune interferon fragment IFN- $\gamma$ (-10) respectively were then added to the wells. Negative control plates were treated with medium alone, whereas for the positive controls, adriamycin was used in a concentration ranging from  $10^{-8}$  to  $10^{-6}$  M. Two days later  $^3$ H-thymidine was added to a final concentration of 1 uCi/well. After further 18 hours of incubation the cells were harvested and thymidine incorporation was determined as described by Carr et al. (*Cell. Immunol.* 5, 23-30 [1972]). It was found that a 14. times lower amount of immune interferon fragment IFN- $\gamma$ (-10) is needed to obtain the same antiproliferative activity on AG 1523 cells as mature recombinant immune interferon (rIFN- $\gamma$ ). Similarly a 5 times, 3 times and 6 times lower amount of IFN- $\gamma$ (-10) was found to be needed to obtain the same antiproliferative activity as rIFN- $\gamma$  on U 937, BS 14 and LLC 1 cells, respectively.

- Immune interferon is known to mediate several immunoregulatory functions. It appears to be one of the macrophage-activating factors (MAF). The macrophage activation was measured in two different ways. Normal human macrophages obtained according to Talmadge et al. (*Eur. J. Immunol.* 16, 1471-1477 [1986]), were activated by the different forms of immune interferon described in the present application. The oxidative burst was measured in terms of enhancement of peroxide release as described by Pick et al. (*J. Immunol. Methods* 45, 211-226 [1981]), whereas the induction of the bactericidal activity of the macrophages was measured as described by Pech et al. (*J. Immunol. Methods* 77, 131-140 [1985]). Since immune interferon exerts its immunoregulatory function via binding to specific receptors, the im-

munoregulatory activities are dependent on the binding of the immune interferon to its receptor. The competitive binding of radioactively labeled mature recombinant immune interferon and recombinant immune interferon fragments (Kung et al., Meth. in Enzymology 119, 296-301 [1986]) to the interferon receptor was measured as described by Rashidbaigi et al. (J. Biol. Chem. 260, 8514-8519 [1985]). The results of the experiments mentioned above are shown in Figure 13 in summarized form. In addition to the remarkably higher antiviral activity of the immune interferon fragments IFN- $\gamma$ (-8), IFN- $\gamma$ (-9), IFN- $\gamma$ (-10) and IFN- $\gamma$ (-11), these immune interferon fragments showed also a remarkably increased macrophage activation and receptor binding capacity.

Exposure of the novel recombinant immune interferon fragments to ambient temperature for several days did not generate new peaks in the HPLC analysis as was observed in the case of the full-length mature recombinant immune interferon and therefore in addition to their higher activity these fragments are more stable.

The purified recombinant immune interferon fragments of the present invention or mixtures thereof can be used for the preparation of pharmaceutical compositions. These pharmaceutical compositions contain a significantly reduced amount of said interferon fragments(s), as compared to the amount of mature recombinant human immune interferon needed to obtain essentially the same antiviral activity and a physiological compatible carrier. This reduced amount is based on the significant higher specific antiviral activity (in units/mg) displayed by the recombinant immune interferon fragments (see Table 1). For the preferred recombinant immune interferon fragments of the present invention this reduced amount was calculated using the values for the specific antiviral activity given in Table 1. The reduced amount is about:

23% for recombinant immune interferon fragment IFN- $\gamma$ (-6)

16% for recombinant immune interferon fragment IFN- $\gamma$ (-8)

14% for recombinant immune interferon fragment IFN- $\gamma$ (-11)

compared to the amount of mature recombinant immune interferon needed (taken as 100%), to obtain the same specific antiviral activity. Similar results may be obtained using the data of Table 2.

The pharmaceutical compositions can be made up in any conventional form including: a) a solid form for oral administration such as tablets, capsules, pills, powders, granules, and the like; b) a liquid form for oral administration such as solutions, syrups, suspensions, elixirs and the like; c) preparations for parenteral administration such as sterile solutions, suspensions or emulsions; and d) preparations for topical administrations such as solutions, suspensions, ointments, creams, gels, micronized powders, aerosols and the like. The pharmaceutical preparations may be sterilized and/or may contain adjuvants such as preservatives, stabilizers, wetting agents, emulsifiers, salts for varying the osmotic pressure and/or buffers.

Parenteral dosage forms may be infusions or injectable solutions which can be injected intravenously or intramuscularly. These preparations can also contain other medicinally active substances. Additional additives such as preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

In accordance with this invention pharmaceutical compositions containing a recombinant immune interferon fragment can be used for the treatment of viral infections, of neoplastic diseases or of rheumatoid arthritis.

It may be administered in pharmaceutically acceptable oral, injectable or topical composition and modes. Dosage and dose rate may parallel that currently being used in clinical applications of the known interferons, typically about  $10^6$  units daily. These pharmaceutical compositions of the invention contain said recombinant immune interferon fragments in association with a compatible pharmaceutically acceptable carrier material. Any conventional carrier material can be utilized. The carrier material can be an organic or inorganic inert carrier material suitable for enteral, percutaneous or parenteral administration. Suitable carriers include water, gelatin, gum arabic, lactose, starch, magnesium stearate, talc, vegetable oils, polyalkylene-glycols, petroleum jelly and the like. Furthermore, the pharmaceutical preparations may contain other pharmaceutically active agents. Additional additives such as flavoring agents, preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

Having now generally described this invention, the same will become better understood by reference to the specific examples which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified.

## 55 Example 1

### A. Principles

pDS8/RBSII, SphI was chosen for the expression of mature recombinant human immune interferon and the recombinant immune interferon fragments of the present invention. For efficient expression the above mentioned vector contains as an expression control sequence the regulatable promoter/operator element  $P_{N25X_{10}}$  (Stüber et al., EMBO J. 3, 3143-3148 [1984]) and the ribosomal binding site RBSII, SphI. This ribosomal binding site has been derived via DNA-synthesis matching the ribosomal binding site under control of E.coli phage T5 promoter  $P_{G25}$  (R. Gentz, Ph.D. Thesis, University of Heidelberg, FRG [1984]). Due to the high efficiency of these expression signals the above mentioned vector can only be stably maintained in E.coli if the promoter/operator element is repressed via binding of the lac repressor to the operator entity of  $P_{N25X_{10}}$ . The lac repressor is encoded by the lacI gene.  $P_{N25X_{10}}$  is efficiently repressed only if sufficient amounts of repressor molecules are present. Therefore the lacI<sup>q</sup> allele was used, which contains a mutant promoter resulting in an increased expression of the repressor gene. The lacI<sup>q</sup> allele is contained in plasmid pDMI,1 (Fig. 4). This plasmid carries in addition to the lacI gene the neo-gene, conferring resistance to kanamycin as a selective marker. Plasmid pDMI,1 is compatible with the expression vector pDS8/RBSII, SphI. E.coli cells to be transformed with expression vectors based on pDS8/RBSII, SphI have to harbour pDMI,1 to ensure stable maintenance of the expression vector. Induction of expression in this system is easily achieved by addition of IPTG to the medium at the desired cell density.

#### B. Plasmid pDS8/RBSII, SphI

The part of pDS8/RBSII, SphI (Fig. 2) between the sites for restriction endonucleases XbaI and XhoI containing the region required for DNA replication and maintenance of the plasmid in the cell and the entire gene for  $\beta$ -lactamase conferring resistance to ampicillin is derived from plasmid pBR322 (Bolivar et al., Gene 2, 95-113 [1977]; Sutcliffe supra). The remaining part of the plasmid carries the regulatable promoter/operator element  $P_{N25X_{10}}$  (Stüber et al., supra) followed by the ribosomal binding site RBSII, SphI which is part of an EcoRI-BamHI fragment, by the coding region for dihydrofolate reductase of mouse AT-3000 cell line (Chang et al., Nature 275, 617-624 [1978]; Masters et al., Gene 21, 59-63 [1983]), by the terminator  $t_0$  of Escherichia coli phage Lambda (Schwarz et al., Nature 272, 410-414 [1978]), by the promoter-free gene for chloramphenicol acetyltransferase (Marcoli et al., FEBS Letters 110, 11-14 [1980]) and by the terminator T1 of the Escherichia coli rrnB operon (Brosius et al., J.Mol.Biol. 148, 107-127 [1981]).

#### C. Plasmid pDMI,1

Plasmid pDMI,1 (Fig. 4) carries the gene for neomycin phosphotransferase from transposon Tn5 (Beck et al., Gene 19, 327-336 [1982]) conferring resistance to kanamycin and the lacI gene (Farabaugh, Nature 274, 765-769 [1978]) with the promoter mutation I<sup>q</sup> (Calos, Nature 274, 762-765 [1978]) encoding the lac-repressor. Furthermore, pDMI,1 contains a region of plasmid pACYC194 (Cao et al., J.Bacteriol. 134, 1141-1156 [1977]) carrying the information necessary for its replication and stable maintenance in E.coli.

#### Example 2

##### Construction of plasmid pGLS encoding IFN- $\gamma$

##### A. Principles

Using chemically synthesized oligonucleotides the IFN- $\gamma$  gene was adapted to the ribosomal binding site RBSII, SphI (Fig. 5). The fragment encoding IFN- $\gamma$  was isolated and ligated into pDS8/RBSII, SphI resulting in plasmid pGLS (Fig. 7).

##### B. Synthesis of the synthetic oligonucleotide fragments adapting the IFN- $\gamma$ gene to RBSII, SphI.

The synthetic oligonucleotide fragments are shown in Fig. 6. The oligonucleotide fragments themselves were prepared simultaneously on a multisynthesizer as described in European Patent Application No. A1 181 491 published on 21.1.86 using controlled pore glass (CPG) as support material (Kiefer et al., Immunol. Meth. 3, 69-83 [1985]; Späth et al., Tetrahedr. Lett 24, 5771-5774 [1983]; Adams et al., J. Am. Chem. Soc. 105, 661-663 [1983]). The tyophilised oligonucleotides were dissolved in water for 1 hour at 4°C to give a DNA concentration of 1  $\mu$ mol/ml.

150 pmol of each oligonucleotide were labelled with 1  $\mu$ l of [<sup>32</sup>P]-ATP (p.r. 3000, 5000 Ci/mmol), 1 U

T4-polynucleotide kinase (Gibco-BRL, Basle) in 10  $\mu$ l 50mM Tris-HCl pH 8.5, 10 mM MgCl<sub>2</sub>, for 10 min at 37°C. All reactions were initiated by the addition of 1  $\mu$ l of 5mM ATP. The reactions were stopped by heating the samples for 7 min at 65°C.

#### 5 C. Construction and isolation of fragment 1 encoding IFN- $\gamma$

4  $\mu$ g of plasmid pRc33FI-900 (European Patent Application No. A2 99 064 published on 25.1.84) with a DNA concentration of 60  $\mu$ g/ml were digested with 10 U of NdeI in BRL "core buffer" (50 mM Tris-HCl, pH 8, 10 mM MgCl<sub>2</sub>, 50 mM NaCl) supplied by Gibco-BRL in Basle, for 1 h at 37°C in a total volume of 20  $\mu$ l. After digestion the DNA was once phenol extracted, treated once with ether, precipitated with ethanol and the pellet dried for 2 minutes in a Speed-vac concentrator. The pellet was dissolved in T4-ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 500  $\mu$ M ATP, 25 pmoles of the phosphorylated oligonucleotides comprising the SphI-NdeI adapter 1 (Fig. 6) in 1 x ligase buffer were added to a final volume of 25  $\mu$ l. Ligations were carried out at 22°C for 3 hours using 1  $\mu$ l DNA-ligase (1 Weiss-unit, Boehringer, Mannheim). The ligation was stopped by incubating the sample for 7 min at 65°C. The DNA was precipitated with ethanol, dried as described above and then resuspended in 50  $\mu$ l NcoI digestion buffer (50 mM Tris-HCl, pH 8, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 50 mM KCl), 10 U NcoI (PRL-Gibco, Basle) were added and the sample was incubated for 1 hr at 37°C. The restriction enzyme was inactivated by heating the sample for 7 min at 65°C. After a phenol extraction the DNA was precipitated and dried as described above. The pellet was dissolved in 20  $\mu$ l Klenow-buffer (50 mM Tris-HCl, pH 7.2, 10 mM MgSO<sub>4</sub>, 100  $\mu$ M DTT) and dATP, dGTP, dCTP and dTTP were added to a final concentration of 100  $\mu$ M (total reaction volume: 30  $\mu$ l). Klenow-enzyme (1 unit, Boehringer Mannheim) was added and the sample was incubated for 1 hr at 22°C. The reaction was stopped by adding 2  $\mu$ l of 0.25M EDTA. The mixture was extracted once with phenol and the DNA was precipitated as described above and then resuspended in SphI-digestion buffer (50 mM Tris-HCl, pH 7.5, 6mM MgCl<sub>2</sub>, 50 mM NaCl, 6mM 2-mercaptoethanol). After addition of 10 U SphI (BRL-Gibco, Basle) the sample was incubated for 1 h at 37°C. The digestion was stopped by heating the sample for 7 min at 65°C, followed by one phenol extraction. The DNA was now precipitated and dried as described above.

The DNA-pellet was dissolved in 10  $\mu$ l gel loading buffer and the DNA fragments electrophoretically separated on a 6% polyacrylamide gel containing 0.5 x Tris-acetate-buffer (40 mM Tris-HCl), 20 mM sodium acetate, 2 mM EDTA, pH 7.8). After staining with ethidiumbromid (0.5  $\mu$ g/ml) the bands were visualized under 300 nm UV-light. The DNA-band encoding the IFN- $\gamma$  gene was cut out of the gel with a scalpel. Marker DNA was phage  $\phi$ X digested with HaeIII (Gibco-BRL, Basle). The gel piece was transferred in a 4x4 mm well in an 0.7% agarose gel containing TBE-buffer (90 mM Tris-borate, 90 mM boric acid, 3 mM EDTA, pH 8.3). The well was sealed with liquid 0.7% agarose in 1xTBE in order to give a homogenous electrical field. In front of the sample a piece of NA45 membrane (Schleicher and Schuell, Dassel, BRD) was inserted and the DNA was electrophoresed on the membrane for 5 min at 300 V. The strip with the DNA was then washed with distilled water and transferred into an Eppendorf tube containing 250  $\mu$ l of 1.5M lithium acetate, 50 mM Tris-HCl pH 8.0 and 10 mM EDTA. The DNA was eluted for 20 min at 65°C with occasional vortexing. The membrane strip was removed from the tube and the sample was once extracted with 200  $\mu$ l phenol that was saturated with 1M Tris pH 8.0. After spinning the samples for 10 min at 12.000 rpm in a microcentrifuge the supernatant containing the DNA was withdrawn. The DNA was precipitated on dry ice for 10 min after adding 20  $\mu$ l of 5M lithium acetate and 440  $\mu$ l isopropanol. The DNA was pelleted for 10 min at 12.000 rpm in a microcentrifuge. The pellet was washed with 80% ethanol and dried in vacuo. The pellet was dissolved in 10  $\mu$ l of TE-buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA).

#### D. Preparation of plasmid pDS8/RBSII,SphI

2 pmoles of plasmid pDS8/RBSII,SphI (400  $\mu$ g/ml) were digested with 10 U SphI in "core buffer" at 37°C in a volume of 50  $\mu$ l for 1 hr. 1 U Scal was added and the digestion continued for 30 min. After digestion the sample was once phenol extracted, treated with ether, precipitated and dried as described above. The pellet was dissolved in 20  $\mu$ l buffer (50 mM Tris-HCl, pH 8) containing 1 unit calf intestinal phosphatase (CIP, Boehringer, Mannheim) and incubated for 1 hour at 37°C. After heat-inactivation of the enzyme and removal of the protein (see above) the DNA was precipitated with ethanol. After resuspension of the DNA, the Scal/SphI fragment of pDS8/RBSII,SphI containing parts of the cat gene, terminator T1, the replication origin, the bla gene, the promoter P<sub>N25X10</sub> and the ribosomal binding site RBSII,SphI was isolated via electrophoresis in 1% agarose gels and electrophoretically transferred onto NA45 membranes. Subsequent elution, ethanol precipitation and resuspension in 100  $\mu$ l TE-buffer was done as described above.

About 1 pmol of the vector fragment was obtained.

#### E. Assembly of plasmid pGLS

- 2 2 µl vector fragment was ligated with 2 µl insert DNA (fragment 1) in ligase buffer (see above) containing 1 µl DNA-ligase (1 Weiss-unit, Boehringer, Mannheim) in a total volume of 20 µl. Ligations were carried out at 22 °C for 3 hours. A control ligation with no insert DNA added was done in parallel. The ligations were stopped by heating the samples for 7 min at 65 °C. The competent E.coli M15 cells used for the transformation were prepared using the method of Morrison (Methods Enzymol. 58, 326-331 [1979]).
- 10 The ligation mixtures were added to 200 µl thawed competent E.coli M15 cells harbouring plasmid pDMSII. The samples were placed on ice for 30 min followed by a 2 min incubation at 42 °C. After adding 0.5 ml of LB-medium the samples were incubated for 90 min in a 37 °C waterbath. The cells were then centrifuged for 30 sec at 12.000 rpm in a microcentrifuge. The supernatants were withdrawn and the pellets were suspended in 100 µl LB-medium and plated on LB-agar plates containing 100 µg/ml ampicillin plus 25 µg/ml kanamycin. The plates were incubated overnight at 37 °C. After transformation of the control ligation no transformants were obtained. The ligation with fragment 1 gave about 200 transformants. Single colonies were picked with a tooth pick, transferred to a tube containing 10 ml LB-medium with 100 µg/ml ampicillin and 25 µg/ml kanamycin and grown for 12 hours at 37 °C with vigorous shaking. The cells were centrifuged for 10 min at 8000 rpm. Plasmid DNA was extracted according to the method of Birnboim et al., (Nucleic
- 20 Acids Res. 7, 1513-1523, [1979]).

The final pellets were dissolved in 200 µl TE-buffer. 4 µl were digested with SphI and XbaI to release the fragment containing the IFN-γ gene with the terminator T1. All DNA samples analysed released the fragment of the expected size of about 1 kb. These plasmids were named pGLS.

#### 25 F. Sequence analysis of the IFN-γ gene, cloned in pGLS

- To confirm that the correct IFN-γ sequence is integrated in pGLS, the double-stranded circular plasmid-DNA was sequenced using a primer endlabeled with [ $\gamma$ -<sup>32</sup>P]-ATP. The primer contains the nucleotides from position 199-218 of pDMSII, SphI and ends therefore 6 nucleotides in front of the ATG of the SphI-site.
- 30 15 µl of the isolated DNA (0.3 pmol) were precipitated with ethanol and washed on a with 80% ethanol. After drying the pellet for 2 min in a Speed vac concentrator the pellet was dissolved in 8 µl 1/4 TE-buffer. After the addition of 2 pmol of the primer, endlabeled with [ $\gamma$ -<sup>32</sup>P]-ATP, the sample was heated for 5 min at 95 °C and then placed for 5 min in a 42 °C water bath. Now for sequencing of the fragment essentially the dideoxy-chain-termination method described by Sanger et al. (Proc. Natl. Acad. Sci. USA. 74, 5463-5467
- 35 [1977]) was used with the modification that since the primer was radioactively labeled, unlabeled deoxyribonucleotide triphosphates (dNTPs) were used in the elongation reaction.

The sequence thus proved that the immune interferon gene was properly integrated into plasmid pGLS.

#### Example 3

- 40 Construction of plasmids pIFN-γ(-6), pIFN-γ(-7), pIFN-γ(-8), pIFN-γ(-9), pIFN-γ(-10) and pIFN-γ(-11)

##### A. Principles

- 45 Chemically synthesized oligonucleotides containing translational stop codons at desired positions were ligated to the unique BamHI recognition site of the IFN-γ gene (Fig. 8) resulting in fragments F(-6), F(-7), F(-8), F(-9), F(-10) and F(-11). These fragments were ligated into pDMSII, SphI resulting in the plasmids pIFN-γ(-6), pIFN-γ(-7), pIFN-γ(-8), pIFN-γ(-9), pIFN-γ(-10) and pIFN-γ(-11), (Fig. 9).

##### 50 B. Preparation of fragments F(-6), F(-7), F(-8), F(-9), F(-10) and F(-11)

- 3 pmoles of plasmid pGLS were digested with 15 U HinfI (Fig. 8) in "cocktail" at 37 °C in a volume of 50 µl for 1 hour. After digestion the restriction enzyme was inactivated. At 65 °C, the sample was phenol extracted, treated with ether, precipitated and dried as described above. The pellet was
- 65 dissolved in 5 µl ligation-buffer. The oligonucleotides comprising adaptors A(-6), A(-7), A(-8), A(-9), A(-10) and A(-11) were synthesized as described in example 2B. 100 pmoles of the synthesized adaptors were added in parallel to 1 µl of the HinfI digested plasmid pGLS. After 10 min in ligation buffer and 1 U of T4-DNA-ligase the reactions were performed in a total volume of 25 µl at 22 °C for 16 hours. The reactions

were stopped by heating the samples for 7 min at 65 °C. After ethanol precipitation the DNA pellets were resuspended in "core buffer" and digested with 15 U EcoRI and 20 U HindIII for 2 hours at 37 °C in a volume of 50 µl. After heat inactivation, phenol extraction, treatment with ether and ethanol precipitation, the samples were dissolved in 10 µl gel loading buffer and electrophoresed on a 5% polyacrylamide gel. The DNA bands were visualized under 300 nm UV-light. The bands containing the IFN-γ genes were cut out of the gel and electrophoresed on a NA45 membrane as described above. The marker DNA used was phage ØX-DNA digested with HaeIII. The DNA's were eluted as described and named F(-6), F(-7), F(-8), F(-9), F(-10) and F(-11) respectively.

#### 10 C. Preparation of plasmid pDS3/RBSII, SphI

As outlined in Fig. 9, clones of plasmid pDS3/RBSII, SphI were digested with 10 U EcoRI and 10 U HindIII for 1 hour at 37 °C in a total volume of 50 µl. After heat inactivation of the enzyme and ethanol precipitation the DNA-pellet was dissolved in 10 µl gel loading buffer. After electrophoresis in a 1% agarose gel the HindIII/EcoRI fragment containing terminator t<sub>0</sub>, the cat gene, terminator T1, the origin of replication, the bla gene and the promoter P<sub>M25X10</sub> was cut out of the gel and eluted as described. The final DNA-pellet was dissolved in 50 µl ligase buffer.

#### 20 D. Assembly of plasmids pIFN-γ(-6), pIFN-γ(-7), pIFN-γ(-8), pIFN-γ(-9), pIFN-γ(-10) and pIFN-γ(-11)

10 µl of the vector fragment and one half of the amount of each isolated fragments F(-6), F(-7), F(-8), F(-9), F(-10) and F(-11) obtained in Example 3B were ligated in parallel in a volume of 20 µl with 1 unit T4-ligase at 22 °C for 3 hours. A control ligation with no insert DNA added was done in parallel. The ligation were stopped by heating the samples for 7 min at 65 °C.

25 Transformations were carried out as described by Morrison (supra) using E.coli M15 harbouring plasmid pDML1. The cells were plated on LB-agar plates containing 100 µg/ml ampicillin and 25 µg/ml kanamycin as described above. The plates were incubated overnight at 37 °C.

As expected no transformants were obtained in the control ligations. The ligations containing vector DNA plus the fragments gave about 500-1000 colonies. Single colonies were picked with a tooth pick and grown in 10 ml LB-medium as described. Plasmid DNA was extracted according to the method of Birnboim et al., (supra). The final pellets were dissolved in 200 µl TE-buffer. 4 µl of the isolated DNA's were digested with 2 U EcoRI and 2 U HindIII. All clones tested released a fragment of the expected size of about 450 bp.

The plasmids containing fragment F(-6) were named pIFN-γ(-6), whereas the plasmids harbouring F(-7), F(-8), F(-9), F(-10) and F(-11) were named pIFN-γ(-7), pIFN-γ(-8), pIFN-γ(-9), pIFN-γ(-10) and pIFN-γ(-11), respectively. These plasmids are expression vectors for the genes coding for the recombinant immune interferon fragments IFN-γ(-6), IFN-γ(-7), IFN-γ(-8), IFN-γ(-9), IFN-γ(-10) and IFN-γ(-11), respectively (Fig. 10).

#### 30 Example 4

##### 40 Expression of IFN-γ genes in E.coli

##### A. Principle

45 In order to demonstrate that the modified IFN-γ genes are expressed in E.coli in high amounts, they were expressed in E.coli M15 (pDML1) transformed with either pIFN-γ(-6), pIFN-γ(-7), pIFN-γ(-8), pIFN-γ(-9), pIFN-γ(-10) or pIFN-γ(-11) and the total cellular protein was analysed by SDS-polyacrylamide gel electrophoresis.

##### 50 B. Production of IFN-γ-proteins in E.coli

E.coli M15 cells containing plasmid pDML1 were transformed with either pIFN-γ(-6), pIFN-γ(-7), pIFN-γ(-8), pIFN-γ(-9), pIFN-γ(-10) or pIFN-γ(-11) and in parallel with pGLS as a control. The transformants were grown in LB-medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. At an optical density at 600 nm of about 0.7 the cultures were induced with IPTG (final concentration 1 mM). After additional 6 hours of incubation the cells were harvested by centrifugation.

##### C. Visualization of proteins produced in E.coli

Cells from : : of 40  $\mu$ l of the cultures were resuspended in sample buffer containing 3% SDS, 3%  $\beta$ -mercaptoethanol, 20% glycerol and 125 mM Tris-HCl, pH 6.8. The samples were boiled for 5 minutes, chilled on ice, centrifuged at 12'000  $\times$  g for 30 seconds and electrophoresed on a SDS-polyacrylamide gel (15.5% acrylamide, ratio of acrylamide/bisacrylamide 1:0.07) according to the procedure described by Laemmli (Nature 227, 680-685, [1970]). After staining of the proteins with Coomassie Brilliant Blue R-250 the unbound dye was removed from the gel. The gel showed that the recombinant immun-interferon fragments IFN- $\gamma$ (-6), IFN- $\gamma$ (-7), IFN- $\gamma$ (-8), IFN- $\gamma$ (-9), IFN- $\gamma$ (-10) and IFN- $\gamma$ (-11) are produced in *E. coli* in higher amounts than the full-length recombinant natural immune interferon, despite the fact that all proteins are under the control of the same expression control sequence.

### Example 5

### Purification of the eluant binant immune interferon fragments

15 All recombinant *E. coli* strains containing an expression vector for a recombinant immune interferon fragment were grown as described above. After induction with IPTG and additional 6 hours incubation the cells were centrifuged at 4000 x g, 10 min, 4 °C. The supernatant was discarded. Each fraction of the cells (100 g) was performed with 0.7M guanidine-hydrochloride in 0.01M sodium borate buffer pH 8.0 (300 ml). The resulting suspension was stirred for 1 hour at 4 °C. The solubilized recombinant immune interferon fragment was separated from the insoluble particles by centrifugation (10'000 x g, 30 min, 4 °C). The supernatant was diluted with 10-fold volume of 0.15M sodium borate pH 8.0 and the remaining particles were sedimented by centrifugation as before. 360 g silica gel 100 (particle size 0.053-0.20 mm) was added to the crude extract. The suspension was stirred gently to avoid sedimentation. After 1 hour, the stirring was stopped. Upon formation of a sediment the supernatant was discarded and the sediment transferred to a column (Ø 5 cm, length 17.5 cm). The silica gel in the column was washed with 0.1M NaCl in 0.01M sodium borate buffer pH 8.0 (flow-rate 6 ml/min). The recombinant immune interferon fragment was eluted with 0.5M tetramethylammonium chloride and 0.7M ammonium sulfate in 0.01M sodium borate buffer pH 8.0 (flow-rate 6 ml/min). The eluate was immediately pumped on a Phenyl-Sepharose CL-4B column (Ø 5 cm, length 21 cm, Pharmacia, Prototyp gel KK33904) which had been equilibrated with 0.5M tetramethylammonium chloride and 0.7M ammonium sulfate in 0.01M sodium borate buffer pH 8.0 (flow-rate 6 ml/min). The recombinant immune interferon fragment was found in the flow through whereas the contaminating proteases were adsorbed by the matrix. The column was regenerated by washing the matrix with 0.01M sodium borate buffer pH 8.0. In a next step the recombinant immune interferon fragment eluted from the Phenyl-Sepharose column was adsorbed on a Phenyl-Sepharose CL-4B column (Ø 5 cm, length 21 cm, Pharmacia) equilibrated with 0.7M ammonium sulfate in 0.01M sodium borate buffer pH 8.0. The column was washed with 0.01M sodium borate buffer pH 8.0 and the recombinant immune interferon fragment was eluted with a 4 hour linear gradient of 0.01M to 0.6M sodium borate buffer pH 8.0. The eluate was diluted with 10-fold volume of water and applied on a Superose 6 (HR 10/30) column (Ø 2.6 cm, length 1 m, Pharmacia) equilibrated with pyrogen free 0.01M sodium phosphate buffer pH 8.0 (flow-rate 4 ml/min). The recombinant immune interferon fragment was eluted using a linear NaCl-gradient of 0-0.6M NaCl. The purified fraction of purification step was applied on a Superose 6 (HR 10/30) column (Ø 2.6 cm, length 1 m, Pharmacia) equilibrated with 0.05M sodium phosphate buffer pH 8.0 (pyrogenfree). The flow-rate was adjusted to 2 ml/min.

45 The purified mature interferon fragments purified in this way were found to be homogeneous upon RP-18 HPLC and SDS-polyacryl amide gel electrophoresis. For C-terminal amino acid analysis the purified protein was digested with *Staphylococcus aureus* V8 protease. The terminal peptides were isolated on RP-18 HPLC, lyophilized, hydrolyzed in HCl and subjected to amino acid analysis. The expected peptides were found in the hydrolysate.

50 After the recombinant lymphine interferon fragment was stable for several  
month up to 10°C.

## Claims

**Claims for the following Contracting States:** BE, CH, DE, FR, GB, IT, LI, NL, PT, SE, SI, TR, UK, JP, KR, AU, NZ, CA, BR, AR, CL, CO, EC, ES, HU, IL, IN, IS, LU, MC, PL, RO, RU, SK, UA, US, VE, ZA, and others.

1. A recombinant immune interferon fragment exhibiting a viral activity higher than that of a recombinant immune interferon and having the amino acid sequence

X-Y-Asp-Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-  
 Asn-Ala-Gly-His-Ser-Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-  
 Gly-Ile-Leu-Lys-Asn-Trp-Lys-Glu-Glu-Ser-Asp-Arg-Lys-Ile-Met-  
 Gln-Ser-Gln-Ile-Val-Ser-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-  
 5 Lys-Asp-Asp-Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-  
 Asp-Met-Asn-Val-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-  
 Asp-Phe-Glu-Lys-Leu-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-  
 Gln-Arg-Lys-Ala-Ile-His-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-  
 Ser-Pro-Ala-Ala-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Z

wherein either X is a methionine and Y is a glutamine residue, or X is hydrogen and Y is a glutamine or a pyroglutamate residue, and Z is

Ser,  
 Ser-Gln,  
 15 Ser-Gln-Met,  
 Ser-Gln-Met-Leu,  
 Ser-Gln-Met-Leu-Phe or  
 Ser-Gln-Met-Leu-Phe-Arg.

- 20 2. A homogeneous recombinant immune interferon fragment according to claim 1 wherein X is a methionine and Y is a glutamine residue.
3. A homogeneous recombinant immune interferon fragment according to claim 1 or 2 wherein Z is Ser-  
 25 Gln-Met-Leu-Phe-Arg.
4. A homogeneous recombinant immune interferon fragment according to claim 1 or 2 wherein Z is Ser-  
 Gln-Met-Leu-Phe.
- 30 5. A homogeneous recombinant immune interferon fragment according to claim 1 or 2 wherein Z is Ser-  
 Gln-Met-Leu.
6. A homogeneous recombinant immune interferon fragment according to claim 1 or 2 wherein Z is Ser-  
 Gln-Met.
- 35 7. A homogeneous recombinant immune interferon fragment according to claim 1 or 2 wherein Z is Ser-  
 Gln.
8. A homogeneous recombinant immune interferon fragment according to claim 1 or 2 wherein Z is Ser.
- 40 9. A homogeneous recombinant immune interferon fragment according to any one of claims 1 to 8 in form  
 of a dimer, a trimer or a tetramer.
10. A replicable microbial expression vehicle comprising a nucleotide sequence, coding upon expression  
 45 for a recombinant immune interferon fragment as defined in any one of claims 1 to 8, which nucleotide  
 sequence is operably linked to an expression control sequence.
11. A replicable microbial expression vehicle according to claim 10 which is a plasmid which contains  
 inserted into the EcoRI and HindIII cleavage sites of pDS8/RBSII, SphI (DSM 3809) a nucleotide  
 50 sequence corresponding to the amino acid sequence defined in claim 3, 5 or 8.
12. A replicable microbial expression vehicle according to claim 10 which is a plasmid which contains  
 inserted into the EcoRI and HindIII cleavage sites of pDS8/RBSII, SphI (DSM 3809) a nucleotide  
 sequence corresponding to the amino acid sequence defined in claim 4, 6 or 7.
- 55 13. A microorganism transformed with a replicable microbial expression vehicle as claimed in any one of  
 claims 10 to 12, comprising a nucleotide sequence coding upon expression for a recombinant immune  
 interferon fragment according to any one of claims 1 to 8.



14. A recombinant immune interferon fragment according to any one of claims 1 to 9 for the treatment of disease states.
15. A process for the preparation of a recombinant immune interferon fragment according to any one of claims 1 to 9 which process comprises causing a culture of a microorganism transformed with a replicable expression vehicle comprising a nucleotide sequence coding upon expression for the said interferon fragment, to grow up and express said interferon fragment and recovering it.
16. A process for the preparation of a microorganism according to claim 10 which process comprises transforming a microorganism with a replicable microbial expression vehicle according to any one of claims 1 to 12.
17. A pharmaceutical composition comprising a recombinant immune interferon fragment according to any one of claims 1 to 9 and a physiological compatible carrier.
18. A pharmaceutical composition according to claim 17 for parenteral administration.
19. A pharmaceutical composition according to claim 17 for topical application.
20. A pharmaceutical composition as claimed in claim 17 for intranasal application.
21. The use of a homogeneous recombinant immune interferon fragment as claimed in any one of claims 1 to 9 for the manufacture of a pharmaceutical composition for the treatment of disease states, such as viral infections, neoplastic diseases and rheumatoid arthritis.

Claims for the following Contracting States : AT, ES, GR

1. A process for the preparation of a homogeneous recombinant immune interferon fragment exhibiting a specific biological activity higher than mature recombinant immune interferon, comprising the amino acid sequence:  
X-Y-A-Ser-Ile-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Asp-His-Ser-Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-Gly-Ile-Ser-Ile-Asn-Trp-Lys-Glu-Glu-Ser-Asp-Arg-Lys-Ile-Met-Gln-Ser-Ile-Ala-Val-Ser-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-Lys-Asn-Phe-Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-Asp-Asn-Ile-Val-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Lys-Lys-Leu-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-Gln-Ile-Ile-Ile-His-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-Ser-Ile-Ile-Ala-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Z  
wherein X is a methionine and Y is a glutamine residue, or X is a histidine residue and Y is a glutamine or a proline residue, and Z is:  
Ser,  
Ser-Glu,  
Ser-Glu-Ile,  
Ser-Glu-Ileu,  
Ser-Glu-Ileu-Phe or  
Ser-Glu-Ileu-Phe-Arg  
which process comprises causing a culture of a microorganism transformed with a replicable expression vehicle, wherein the transformed microorganism is capable of expressing said interferon fragment, to grow up and express the said interferon fragment and recovering it.
2. A process according to claim 1 characterized in that a culture of a microorganism transformed with a replicable expression vehicle comprising a nucleotide sequence coding for expression for a homogeneous recombinant immune interferon fragment as defined in claim 1 and containing a methionine and a glutamine residue, is used.

3. A process according to claim 1 characterized in that a culture of a microorganism, transformed with a replicable expression vehicle comprising a nucleotide sequence coding upon expression for a homogeneous recombinant immune interferon fragment as defined in claim 1 or 2 wherein Z is Ser-Gln-Met-Leu-Phe-Arg, is used.
4. A process according to claim 1 characterized in that a culture of a microorganism, transformed with a replicable expression vehicle comprising a nucleotide sequence coding upon expression for a homogeneous recombinant immune interferon fragment as defined in claim 1 or 2 wherein Z is Ser-Gln-Met-Leu-Phe, is used.
5. A process according to claim 1 characterized in that a culture of a microorganism, transformed with a replicable expression vehicle comprising a nucleotide sequence coding upon expression for a homogeneous recombinant immune interferon fragment as defined in claim 1 or 2 wherein Z is Ser-Gln-Met-Leu, is used.
6. A process according to claim 1 characterized in that a culture of a microorganism, transformed with a replicable expression vehicle comprising a nucleotide sequence coding upon expression for a homogeneous recombinant immune interferon fragment as defined in claim 1 or 2 wherein Z is Ser-Gln-Met, is used.
7. A process according to claim 1 characterized in that a culture of a microorganism, transformed with a replicable expression vehicle comprising a nucleotide sequence coding upon expression for a homogeneous recombinant immune interferon fragment as defined in claim 1 or 2 wherein Z is Ser-Gln, is used.
8. A process according to claim 1 characterized in that a culture of a microorganism, transformed with a replicable expression vehicle comprising a nucleotide sequence coding upon expression for a homogeneous recombinant immune interferon fragment as defined in claim 1 or 2 wherein Z is Ser, is used.
9. A process according to claim 1 or 2 characterized in that said interferon fragment is recovered in form of a dimer, a trimer or a tetramer.
10. A process for the preparation of a microorganism capable of expressing a recombinant immune interferon fragment as defined in any one of claims 1 to 8 which process comprises transforming a microorganism with a replicable microbial expression vehicle comprising a nucleotide sequence coding upon expression for the said immune interferon fragment, which nucleotide sequence is operably linked to an expression control sequence and growing the transformed microorganism in a culture medium.
11. A method for the preparation of a pharmaceutical composition comprising a homogeneous recombinant immune interferon fragment as defined in any one of claims 1 to 9, which process comprises mixing the said interferon fragment with a compatible pharmaceutically acceptable carrier material.
12. A pharmaceutical composition comprising a homogeneous recombinant immune interferon fragment as defined in any one of claims 1 to 9.
13. The use of a homogeneous recombinant immune interferon fragment as defined in any one of claims 1 to 9 for the manufacture of a pharmaceutical composition for the treatment of various disease states such as viral infections, neoplastic diseases and rheumatoid arthritis.
14. A homogeneous recombinant immune interferon fragment prepared by a process according to any one of claims 1 to 9.
15. A microorganism which is capable of expressing a recombinant human immune interferon fragment as defined in any one of claims 1 to 8 prepared by a process according to claim 10.
16. A replicable microbial expression vehicle comprising a nucleotide sequence, coding upon expression for a recombinant immune interferon fragment as defined in any one of claims 1 to 8, which nucleotide sequence is operably linked to an expression control sequence.

17. A replicable microbial expression vehicle according to claim 16 which contains inserted in the EcoRI and HindIII cleavage sites of pDS8/RBSII, SpH sequence corresponding to the amino acid sequence defined in claim 4, 5.
18. A replicable microbial expression vehicle according to claim 16 which contains inserted in the EcoRI and HindIII cleavage sites of pDS8/RBSII, SpH sequence corresponding to the amino acid sequence defined in claim 4, 6.
19. A microorganism transformed with a replicable microbial expression vehicle according to claim 16 or 17, comprising a nucleotide sequence coding upon expression for an interferon fragment as defined in any one of claims 1 to 8.

# Revendications

Revendication pour les Etats contractants suivants : BE, CH, DE, FR, GB, NL, SE

1. Fragment d'interféron immun recombiné homogène présentant une activité spécifique supérieure à celle de l'interféron immun recombiné mature et ayant la séquence
- X-Y-Asp-Phe-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-  
Asn-Ile-Thr-His-Ser-Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-  
Gly-Ile-Leu-Gln-Asn-Trp-Lys-Glu-Glu-Cys-Asp-Arg-Lys-Ile-Met-  
Gln-Ile-Ser-Val-Ser-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-  
Lys-Phe-Ile-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-  
Asp-Ile-Ile-Gln-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-  
Asp-Ile-Ile-Leu-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-  
Gln-Ile-Ile-Ile-His-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-  
Ser-Phe-Val-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Z
- dans laquelle soit X est méthionine et Y est un résidu de glutamine, soit X est un résidu d'hydrogène et Y est un résidu de glutamine ou de pyroglutamate, et Z est
- Ser.  
Ser.  
Ser.  
Ser-Ile-Ile-Leu,  
Ser-Ile-Ile-Phe ou  
Ser-Ile-Ile-Phe-Arg.
2. Fragment d'interféron immun recombiné homogène selon la revendication 1, où X est méthionine et Y est un résidu de glutamine.
3. Fragment d'interféron immun recombiné homogène selon la revendication 1, où Z est Ser-Gln-Met-Met-Arg.
4. Fragment d'interféron immun recombiné homogène selon la revendication 1, où Z est Ser-Gln-Met-Met-Arg.
5. Fragment d'interféron immun recombiné homogène selon la revendication 1, où Z est Ser-Gln-Met-Met-Arg.
6. Fragment d'interféron immun recombiné homogène selon la revendication 1, où Z est Ser-Gln-Met-Met-Arg.
7. Fragment d'interféron immun recombiné homogène selon la revendication 1, où Z est Ser-Gln-Met-Met-Arg.
8. Fragment d'interféron immun recombiné homogène selon la revendication 1, où Z est Ser.
9. Fragment d'interféron immun recombiné homogène selon l'une quelconque des revendications 1 à 8, sous forme d'un dimère, d'un trimère ou d'un tétramère.

10. Véhicule d'expression répliquable comprenant une séquence nucléotidique codant lors de l'expression pour un fragment d'interféron immun recombiné comme défini dans l'une quelconque des revendications 1 à 9, laquelle séquence nucléotidique est fonctionnellement associée à une séquence de régulation de l'expression.
11. Véhicule d'expression répliquable selon la revendication 10 qui est un plasmide qui contient, insérée entre les sites de clivage EcoR I et Hind III de pDS8/RBSII, SphI (DSM 3033), une séquence nucléotidique correspondant à la séquence des aminoacides définie dans les revendications 3, 5 ou 8.
12. Véhicule d'expression répliquable selon la revendication 10 qui est un plasmide qui contient, insérée entre les sites de clivage EcoR I et Hind III de pDS8/RBSII, SphI (DSM 3033), une séquence nucléotidique correspondant à la séquence des aminoacides définie dans les revendications 4, 6 ou 7.
13. Microorganisme transformé avec un véhicule microbien d'expression répliquable selon l'une quelconque des revendications 10 à 12, comprenant une séquence nucléotidique codant lors de l'expression pour un fragment d'interféron immun recombiné selon l'une quelconque des revendications 1 à 9.
14. Fragment d'interféron immun recombiné selon l'une quelconque des revendications 1 à 9 pour le traitement des états pathologiques.
15. Procédé de préparation d'un fragment d'interféron immun recombiné selon l'une quelconque des revendications 1 à 9, lequel procédé consiste à faire en sorte qu'une culture d'un microorganisme, transformé avec un véhicule microbien d'expression répliquable comprenant une séquence nucléotidique codant lors de l'expression pour ledit fragment d'interféron, se développe et exprime ledit fragment d'interféron.
16. Procédé de préparation d'un microorganisme selon la revendication 13, lequel procédé comprend la transformation d'un microorganisme avec un véhicule microbien d'expression répliquable selon l'une quelconque des revendications 10 à 12.
17. Composition pharmaceutique comprenant un fragment d'interféron immun recombiné selon l'une quelconque des revendications 1 à 9 et un véhicule physiologique compatible.
18. Composition pharmaceutique selon la revendication 17 pour l'administration parentérale.
19. Composition pharmaceutique selon la revendication 17 pour l'administration locale.
20. Composition pharmaceutique selon la revendication 17 pour l'application intranasale.
21. Utilisation d'un fragment d'interféron immun recombiné homogène selon l'une quelconque des revendications 1 à 9 pour la préparation d'une composition pharmaceutique pour le traitement de divers états pathologiques tels que les infections virales, les maladies néoplasiques et la polyarthrite rhumatoïde.

#### Revendications pour les Etats contractants suivants : AT, ES, GR

1. Procédé de préparation d'un fragment d'interféron immun recombiné homogène présentant une activité antivirale spécifique supérieure à celle de l'interféron immun recombiné nature et ayant la séquence d'acides aminés
- X-Y-Asp-Pro-Ile-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Ala-Gly-Ile-Ser-Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-Gly-Ile-Leu-Leu-Asn-Trp-Lys-Glu-Glu-Ser-Asp-Arg-Lys-Ile-Met-Gln-Ser-Gln-Ile-Val-Ser-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-Lys-Asp-Asp-Ile-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-Asp-Met-Asp-Val-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-Lys-Leu-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-Gln-Arg-Lys-Ile-Ile-His-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-Ser-Pro-Ala-Ile-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Z

dan- le résidu X est méthionine et Y est un reste de glutamine, soit Z est un reste d'hydrogène et Y est un reste de glutamine ou de pyroglutamate, ou Z est

Ser,

5

Ser-

Ser-Gln-Met,

Ser-Gln-Met-Leu,

Ser-Gln-Met-Leu-Phe ou

Ser-Gln-Met-Leu-Phe-Arg

10

lequel le processus consiste à faire en sorte qu'une culture d'un microorganisme transformé avec un véhicule d'expression répliquable, lequel microorganisme transformé est capable d'exprimer ledit fragment d'interféron, se développe et exprime ledit fragment d'interféron, la récupération de celui-ci.

15

2. Procédé selon la revendication 1, caractérisé en ce que l'on utilise un microorganisme transformé avec un véhicule d'expression répliquable comprenant une séquence codante spécifique pour un fragment d'interféron immun recombiné dont la séquence est définie dans la revendication 1, où X est méthionine et Y est un reste de glutamine.

20

3. Procédé selon la revendication 1, caractérisé en ce que l'on utilise un microorganisme transformé avec un véhicule d'expression répliquable comprenant une séquence codante spécifique pour un fragment d'interféron immun recombiné dont la séquence est définie dans la revendication 2, où Z est Ser-Gln-Met-Leu-Phe-Arg.

25

4. Procédé selon la revendication 1, caractérisé en ce que l'on utilise un microorganisme transformé avec un véhicule d'expression répliquable comprenant une séquence codante spécifique pour un fragment d'interféron immun recombiné dont la séquence est définie dans la revendication 2, où Z est Ser-Gln-Met-Leu-Phe.

30

5. Procédé selon la revendication 1, caractérisé en ce que l'on utilise un microorganisme transformé avec un véhicule d'expression répliquable comprenant une séquence codante spécifique pour un fragment d'interféron immun recombiné dont la séquence est définie dans la revendication 2, où Z est Ser-Gln-Met-Leu.

35

6. Procédé selon la revendication 1, caractérisé en ce que l'on utilise un microorganisme transformé avec un véhicule d'expression répliquable comprenant une séquence codante spécifique pour un fragment d'interféron immun recombiné dont la séquence est définie dans la revendication 2, où Z est Ser-Gln-Met.

40

7. Procédé selon la revendication 1, caractérisé en ce que l'on utilise un microorganisme transformé avec un véhicule d'expression répliquable comprenant une séquence codante spécifique pour un fragment d'interféron immun recombiné dont la séquence est définie dans la revendication 2, où Z est Ser-Gln.

45

8. Procédé selon la revendication 1, caractérisé en ce que l'on utilise un microorganisme transformé avec un véhicule d'expression répliquable comprenant une séquence codante spécifique pour un fragment d'interféron immun recombiné dont la séquence est définie dans la revendication 2, où Z est Ser.

50

9. Procédé selon la revendication 1 ou 2, caractérisé en ce que ledit fragment d'interféron est récupéré sous forme de dimère, d'un trimère ou d'un tétramère.

55

10. Procédé de préparation d'un microorganisme capable d'exprimer un fragment d'interféron immun recombiné, lequel comprend la transformation d'un microorganisme avec un véhicule microbien d'expression répliquable comprenant une séquence codante spécifique pour un fragment d'interféron immun, laquelle séquence est fonctionnellement associée à une séquence d'expression, et la

multiplié dans un microorganisme transformé dans un milieu de culture.

11. Procédé de préparation d'une composition pharmaceutique comprenant un fragment d'interféron immun recombiné homogène comme défini dans l'une quelconque des revendications 1 à 9, lequel procédé consiste à mélanger dudit fragment d'interféron avec un véhicule compatible pharmaceutiquement.
12. Composition pharmaceutique comprenant un fragment d'interféron immun recombiné homogène comme défini dans l'une quelconque des revendications 1 à 9.
13. Utilisation d'un fragment d'interféron immun recombiné homogène comme défini dans l'une quelconque des revendications 1 à 9 pour la préparation d'une composition pharmaceutique pour le traitement de divers états pathologiques, tels que les infections virales, les maladies néoplasiques et la polyarthrite rhumatoïde.
14. Fragment d'interféron immun recombiné homogène préparé selon le procédé de l'une quelconque des revendications 1 à 9.
15. Microorganisme capable d'exprimer un fragment d'interféron immun humain recombiné comme défini dans l'une quelconque des revendications 1 à 9, préparé selon le procédé de la revendication 10.
16. Véhicule d'expression répliquable comprenant une séquence nucléotidique codant lors de l'expression un fragment d'interféron immun recombiné comme défini dans l'une quelconque des revendications 1 à 9, laquelle séquence nucléotidique est fonctionnellement associée à une séquence d'expression.
17. Véhicule d'expression répliquable selon la revendication 16 qui est un plasmide qui contient, insérée dans une région de clivage EcoR I et Hind III de pDS8/RBSII, SphI (DSM 3613), une séquence nucléotidique correspondant à la séquence des aminoacides définie dans les revendications 3, 5 ou 8.
18. Véhicule d'expression répliquable selon la revendication 16 qui est un plasmide qui contient, insérée dans une région de clivage EcoR I et Hind III de pDS8/RBSII, SphI (DSM 3613), une séquence nucléotidique correspondant à la séquence des aminoacides définie dans les revendications 4, 6 ou 7.
19. Microorganisme transformé avec un véhicule microbien d'expression répliquable selon l'une quelconque des revendications 16 à 18 comprenant une séquence nucléotidique codant lors de l'expression pour un fragment d'interféron immun recombiné, comme défini dans l'une quelconque des revendications 1 à 9.

#### 40 Patentansprüche

Patentsprüche in folgenden Vertragsstaaten: BE, CH, DE, FR, GB, IT, LI, LU, NL, SI

1. Ein homogenes, rekombinantes Immuninterferonfragment mit einer spezifischen antiviralen Aktivität, die grösser ist als die von reifem, rekombinantem Immuninterferon, und das die Aminosäuresequenz

X-Y-Asp-Arg-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-  
Asn-Ala-Gly-Ser-Ser-Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-  
Gly-Ile-Leu-Asn-Trp-Lys-Glu-Glu-Ser-Asp-Arg-Lys-Ile-Met-  
Gln-Ser-Glu-Val-Ile-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-  
Lys-Asp-Asn-Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-  
Asp-Met-Asp-Val-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-  
Asp-Phe-Glu-Lys-Leu-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-  
Gln-Arg-Lys-Ile-Ile-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-  
Ser-Pro-Ala-Ile-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Z

aufweist, wobei X entweder X für Methionin steht und Y ein Glutaminrest ist, oder X für Valin steht und Y für Glutamin- oder ein Pyroglutaminsäurerest steht, und Z für Ser,

Ser-  
 Ser-  
 Ser-  
 Ser-  
 5 Ser-  
 steht

2. Ein rekombinantes Immuninterferonfragment gemäss Anspruch 1, das durch einen Glutaminrest und Y ein Glutaminrest ist.
3. Ein rekombinantes Immuninterferonfragment gemäss Anspruch 1, das durch einen Glutaminrest für Ser-Gln-Met-Leu-Phe-Arg steht.
4. Ein rekombinantes Immuninterferonfragment gemäss Anspruch 1, das durch einen Glutaminrest für Ser-Gln-Met-Leu-Phe steht.
5. Ein rekombinantes Immuninterferonfragment gemäss Anspruch 1, das durch einen Glutaminrest für Ser-Gln-Met-Leu steht.
6. Ein rekombinantes Immuninterferonfragment gemäss Anspruch 1, das durch einen Glutaminrest für Ser-Gln-Met steht.
7. Ein rekombinantes Immuninterferonfragment gemäss Anspruch 1, das durch einen Glutaminrest für Ser-Gln steht.
8. Ein rekombinantes Immuninterferonfragment gemäss Anspruch 1, das durch einen Glutaminrest für Ser steht.
9. Ein rekombinantes Immuninterferonfragment gemäss einem der Ansprüche 1 bis 8 in Form eines Trimers oder eines Tetramers.
10. Ein vektor, mikrobieller Expressionsträger, der eine Nukleotidsequenz enthält, die nach der Expression für ein rekombinantes Immuninterferonfragment gemäss einem der Ansprüche 1 bis 8 kodiert, das durch einen Glutaminrest und Y ein Glutaminrest ist.
11. Ein vektor, mikrobieller Expressionsträger gemäss Anspruch 10, der eine Nukleotidsequenz enthält, die einer Aminosäuresequenz entspricht, wie sie in den Ansprüchen 3, 5 oder 6 angegeben ist und die in die EcoRI and HindIII Schnittstelle von pSphI (DSM 3809) eingebracht ist.
12. Ein vektor, mikrobieller Expressionsträger gemäss Anspruch 10, der eine Nukleotidsequenz enthält, die einer Aminosäuresequenz entspricht, wie sie in den Ansprüchen 4, 6 oder 7 angegeben ist und die in die EcoRI and HindIII Schnittstelle von pSphI (DSM 3809) eingebracht ist.
13. Ein Zellstammus, der mit einem replizierbaren, mikrobiellen Expressionsträger transformiert ist, der eine Nukleotidsequenz enthält, die nach der Expression für ein rekombinantes Immuninterferonfragment gemäss einem der Ansprüche 1 bis 8 kodiert.
14. Ein rekombinantes Immuninterferonfragment gemäss einem der Ansprüche 1 bis 8 zur Behandlung von Krebs.
15. Ein Verfahren zur Herstellung eines rekombinanten Immuninterferonfragments gemäss einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, dass eine Kultur eines Mikroorganismus, der mit einem replizierbaren, mikrobiellen Expressionsträger transformiert ist, der eine Nukleotidsequenz enthält, die bei der Expression ein rekombinantes Immuninterferonfragment kodiert, kultiviert und gewonnen wird.

16. Ein Verfahren zur Herstellung eines Mikroorganismus gemäss Anspruch 10, dadurch gekennzeichnet, dass ein Mikroorganismus mit einem replizierbaren, mikrobiellen Expressionsträger transformiert wird.
17. Eine pharmazeutische Zusammensetzung enthaltend ein rekombinantes Immuninterferonfragment gemäss einem der Ansprüche 1 bis 9 und ein physiologisch verträglicher Träger.
18. Eine pharmazeutische Zusammensetzung gemäss Anspruch 17 zur parenteralen Verabreichung.
19. Eine pharmazeutische Zusammensetzung gemäss Anspruch 17 zur topischen Verabreichung.
20. Eine pharmazeutische Zusammensetzung gemäss Anspruch 17 zur intranasalen Verabreichung.
21. Die Verwendung eines rekombinanten Immuninterferonfragments gemäss einem der Ansprüche 1 bis 9, für die Herstellung einer pharmazeutischen Zusammensetzung zur Behandlung verschiedener Krankheiten, wie z.B. viraler Infektionen, neoplastischen Erkrankungen oder rheumatischer Arthritis.
- Patentsprüche in den Vertragsstaaten : AT, ES, GR
- 20 1. Ein Verfahren zur Herstellung eines homogenen, rekombinanten Immuninterferons mit einer spezifischen biologischen Aktivität, die grösser ist als die von reifem, rekombinantem Interferon, und das durch die Aminosäuresequenz
- 25 X-Y-Asp-Ileu-Val-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Ala-Gly-Ser-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-Gly-Ile-Leu-Ile-Tyr-Phe-Lys-Glu-Glu-Ser-Arg-Lys-Ile-Met-Gln-Ser-Gln-Ile-Gln-Lys-ser-Val-Glu-Thr-Ile-Lys-Glu-Asp-Met-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Ile-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-Gln-Arg-Leu-Ile-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-Ser-Pro-Arg-Lys-Ile-Gly-Lys-Arg-Lys-Arg-Z
- 30 aufweist, wobei X für Methionin steht und Y ein Glutaminrest ist, oder X für ein Pyroglutaminsäurerest steht, und Z für Ser, Ser-Gln, Ser-Gln-Met, Ser-Gln-Met, Ser-Gln-Ileu oder Ser-Gln-Met-Arg steht, dadurch gekennzeichnet, dass eine Kultur eines Mikroorganismus, der mit einem replizierbaren Expressionsträger transformiert ist, und der fähig ist ein obengenanntes Immuninterferonfragment zu exprimieren, beschleunigt wird und das Interferonfragment gewonnen wird.
- 35 2. Ein Verfahren gemäss Anspruch 1, dadurch gekennzeichnet dass eine Kultur eines Mikroorganismus, der mit einem replizierbaren Expressionsträger transformiert ist, der eine Nukleotidsequenz enthält, die bei der Expression ein Immuninterferonfragment kodiert, wie es in Anspruch 1 definiert ist, kodiert, worin X für Methionin steht und Y ein Glutaminrest ist, verwendet wird.
- 40 3. Ein Verfahren gemäss Anspruch 1, dadurch gekennzeichnet dass eine Kultur eines Mikroorganismus, der mit einem replizierbaren Expressionsträger transformiert ist, der eine Nukleotidsequenz enthält, die bei der Expression ein Immuninterferonfragment kodiert, wie es in Anspruch 1 definiert ist und worin Z Ser-Gln-Met-Leu-Phe-Arg steht, verwendet wird.
- 45 4. Ein Verfahren gemäss Anspruch 1, dadurch gekennzeichnet dass eine Kultur eines Mikroorganismus, der mit einem replizierbaren Expressionsträger transformiert ist, der eine Nukleotidsequenz enthält, die bei der Expression ein Immuninterferonfragment kodiert, wie es in Anspruch 1 definiert ist



und die Sequenz für Ser-Gln-Met-Leu-Phe steht, verwendet wird.

5. Ein Mikroorganismus gemäss Anspruch 1, dadurch gekennzeichnet dass eine Kultur, der ein in einem replizierbaren Expressionsträger transformiert ist, der eine Nukleotidsequenz für ein Immuninterferonfragment kodiert, wie es in Anspruch 1 oder 2 definiert ist und die Sequenz für Ser-Gln-Met-Leu-Phe steht, verwendet wird.
6. Ein Mikroorganismus gemäss Anspruch 1, dadurch gekennzeichnet dass eine Kultur, der ein in einem replizierbaren Expressionsträger transformiert ist, der eine Nukleotidsequenz für ein Immuninterferonfragment kodiert, wie es in Anspruch 1 oder 2 definiert ist und die Sequenz für Ser-Gln-Met steht, verwendet wird.
7. Ein Mikroorganismus gemäss Anspruch 1, dadurch gekennzeichnet dass eine Kultur, der ein in einem replizierbaren Expressionsträger transformiert ist, der eine Nukleotidsequenz für ein Immuninterferonfragment kodiert, wie es in Anspruch 1 oder 2 definiert ist und die Sequenz für Ser-Gln steht, verwendet wird.
8. Ein Mikroorganismus gemäss Anspruch 1, dadurch gekennzeichnet dass eine Kultur, der ein in einem replizierbaren Expressionsträger transformiert ist, der eine Nukleotidsequenz für ein Immuninterferonfragment kodiert, wie es in Anspruch 1 oder 2 definiert ist und die Sequenz für Ser steht, verwendet wird.
9. Ein Immuninterferon gemäss Anspruch 1 oder 2, dadurch gekennzeichnet dass das Immuninterferon in Form eines Dimers, eines Trimers oder eines Tetramers gewonnen wird.
10. Ein Verfahren zur Herstellung eines Mikroorganismus, der fähig ist ein Immuninterferonfragment, wie es in einem der Ansprüche 1 bis 8 definiert ist, zu exprimieren, das in einem Mikroorganismus mit einem replizierbaren, mikrobiellen Expressionsträger, der eine Nukleotidsequenz enthält, die bei der Expression für ein oberflächlich gebundenes Immuninterferonfragment kodiert, operabel an eine Expressionsträgersequenz gebunden ist und in einem geeigneten Mikroorganismus in einem Kulturmedium vermehrt wird.
11. Ein Verfahren zur Herstellung einer pharmazeutischen Zusammensetzung, die ein rekombinantes Immuninterferonfragment gemäss einem der Ansprüche 1 bis 8 enthält, dadurch gekennzeichnet, dass ein solches Interferonfragment mit einem passenden Trägerstoff kombiniert wird.
12. Ein pharmazeutisches Präparat, das eine Zusammensetzung enthaltend ein homogenes Immuninterferonfragment, wie es in einem der Ansprüche 1 bis 8 definiert ist.
13. Die Anwendung eines homogenen, rekombinanten Immuninterferonfragments, wie es in einem der Ansprüche 1 bis 8 definiert ist, für die Herstellung einer pharmazeutischen Zusammensetzung zur Behandlung einer Krankheit, wie zum Beispiel viralen Infektionen oder Arthritis.
14. Ein Verfahren zur Herstellung eines rekombinanten Immuninterferonfragments, hergestellt durch ein Verfahren gemäss einem der Ansprüche 1 bis 9.
15. Ein Verfahren, bei dem ein Mikroorganismus, der fähig ist ein rekombinantes menschliches Immuninterferonfragment, wie es in einem der Ansprüche 1 bis 8 definiert ist, zu exprimieren, hergestellt wird, durch ein Verfahren gemäss einem der Ansprüche 1 bis 9.
16. Ein synthetischer, mikrobieller Expressionsträger, der eine Nukleotidsequenz für ein rekombinantes Immuninterferonfragment, wie es in einem der Ansprüche 1 bis 8 definiert ist, enthält, dadurch gekennzeichnet dass die Nukleotidsequenz operabel an eine Expressionssequenz gebunden ist.
17. Ein Verfahren zur Herstellung eines rekombinanten Immuninterferonfragments, hergestellt durch ein Verfahren gemäss einem der Ansprüche 1 bis 9.

Nukleotidsequenz, die einer Aminosäuresequenz entspricht, wie sie in den Ansprüchen 3, 5 oder 8 definiert ist, wobei die Nukleotidsequenz in die EcoRI and HindIII Schnittstelle von pDS8/RBS3 eingebaut ist.

5 18. Ein replizierbarer, mikrobieller Expressionsträger gemäss Anspruch 16, der ein Plasmid enthält, das eine Nukleotidsequenz enthält, die einer Aminosäuresequenz entspricht, wie sie in den Ansprüchen 4, 6 oder 7 definiert ist, wobei die Nukleotidsequenz in die EcoRI and HindIII Schnittstelle von pDS8/RBS3 eingebaut ist.

10 19. Ein Mikroorganismus, der mit einem replizierbaren, mikrobiellen Expressionsträger gemäss einem der Ansprüche 1 bis 18 transformiert ist, der eine Nukleotidsequenz enthält, die bei Expression für ein Interferonfragment, wie es in einem der Ansprüche 1 bis 8 definiert ist, kodiert.

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Fig. 1

ATG CAC GAC CCA TAT GTA AAA GAA GCA GAA AAC G C C AAA TAT  
 (Met) G C C C Tyr Val Lys Glu Ala Glu Asn C C C Lys Tyr

TTT ACC CCA CAT TCA GAT GTA GCG GAT AAT C C C CTT TTC  
 Phe Asn Ala Tyr His Ser Asp Val Ala Asp Asn G C C Leu Phe

TTA GCA AAT TTG AAG AAT TGG AAA GAG GAG AGT G C C AAA ATA  
 Leu G C C Lys Asn Trp Lys Glu Glu Ser C C C Lys Ile

ATG C CCA ATT GTC TCC TTT TAC TTC AAA C C C AAA AAC  
 Met G C C Ile Val Ser Phe Tyr Phe Lys C C C Lys Asn

TTT ACC GAC CAG AGC ATC CAA AAG AGT GTG G C C C ATC AAG  
 Phe L C C Gln Ser Ile Gln Lys Ser Val C C C Ile Lys

GAA G CCA TAT GTC AAG TTT TTC AAT AGC AAT C C C AAA CGA  
 Glu A C C Ala Val Lys Phe Phe Asn Ser Asn C C C Lys Arg

GAT GAC TCG CCA AAG CTG ACT AAT TAT TCG GTG C C C TTG AAT  
 Asp A C C Glu Lys Leu Thr Asn Tyr Ser Val C C C Leu Asn

GTC C CCA GCA ATA CAT GAA CTC ATC CAC C C C GCT GAA  
 Val G C C Lys Ala Ile His Glu Leu Ile G C C Ala Glu

CTG T CCA TCA GCT AAA ACA CGG AAG CGA A C C C CAG ATG  
 Leu S C C Ala Ala Lys Thr Gly Lys Arg Lys C C C Gln Met

CTG T CCA CCA GCA AGA GCA TCC CAG TAA  
 Leu I C C Lys Arg Arg Ala Ser Gln

Fig. 2

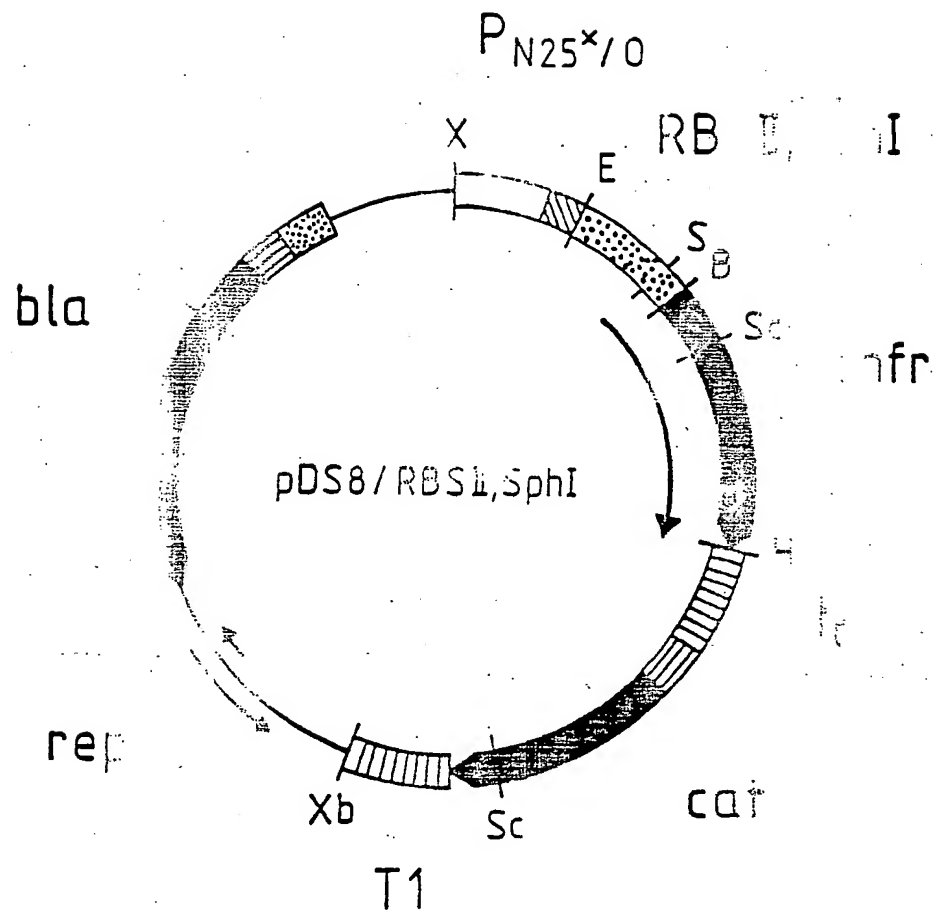


Fig. 3

|      | 10  | 20                | 30                 | 50         |          |          |
|------|-----|-------------------|--------------------|------------|----------|----------|
| 0    | TTT | GGCATCCCTA        | ACATATCCGA         | ATG        | ACAACGGA |          |
| 50   | TTT | ATCCGTTCCG        | ATAGGGTTTG         | AGT        | TATATGCT |          |
| 100  | TTT | CTTTGCTCAA        | AGAATCATAA         | AAA        | CTTTCAGG |          |
| 150  | T   | TTG               | TCTATAATAG         | ATTCAAATTG | TGAG     | AATTTGAA |
| 200  | TAG | ACGAGAAAT         | <u>ATCCATGCCA</u>  | GGAT       | ATGGTTGG |          |
| 250  | TAG | <u>TCCATCGTGG</u> | <u>CCGTGTCCCA</u>  | AAAT       | TGGCAAGA |          |
| 300  | TTT | <u>ACCGTGGCGT</u> | <u>CCGCTCAGCA</u>  | ACG        | ACTTCCAA |          |
| 350  | T   | <u>CAAGCTCTTC</u> | <u>ACTGGAAAGCT</u> | AAAT       | GTGATTAT |          |
| 400  | T   | <u>ACCGGTTTCT</u> | <u>CAATTCCTGA</u>  | CAAT       | TTTAAAGG |          |
| 450  | T   | <u>TATATTTTTC</u> | <u>ACTACAGAAC</u>  | TCA        | CAGGAGGA |          |
| 500  | T   | <u>TTCCGAATAT</u> | <u>TTGGATGAT</u>   | CCC        | ATTGAACA |          |
| 550  | T   | <u>CCATGTAA</u>   | <u>TAGCATGCT</u>   | TTG        | AGGCAGTT |          |
| 600  | T   | <u>CCATGGCATG</u> | <u>ATGACCGAG</u>   | CCC        | TCTTTGTG |          |
| 650  | T   | <u>TCTAGGAAT</u>  | <u>TCAAAATGAC</u>  | ACCT       | GAAATTGA |          |
| 700  | T   | <u>TTTAAATTT</u>  | <u>TTTCAGATA</u>   | CCC        | CTCTGAGG |          |
| 750  | T   | <u>AAAAGGG</u>    | <u>TTATATCT</u>    | TTG        | GAAGAAA  |          |
| 800  | T   | <u>AGATGCTTC</u>  | <u>CAAGTTCTCT</u>  | GCT        | AGCTATG  |          |
| 850  | T   | <u>ATAATATAG</u>  | <u>ATTTTCTCT</u>   | GCT        | CCCAAGCT |          |
| 900  | T   | <u>TTATAGAT</u>   | <u>CAATATCTCT</u>  | GCT        | TCTGGATT |          |
| 950  | T   | <u>GCTCGGTTTC</u> | <u>CCCCGCTCT</u>   | TTT        | AGAATCCA |          |
| 1000 | T   | <u>GCGAGATTTT</u> | <u>CAAGAGCTTA</u>  | GGA        | AGAGAAAA |          |
| 1050 | T   | <u>ATATACGAG</u>  | <u>GTGATATTT</u>   | CCC        | TTAAAGAA |          |
| 1100 | T   | <u>CATTCGAT</u>   | <u>ATCTGCTCA</u>   | TGA        | CAGACCGT |          |
| 1150 | T   | <u>ATACGCT</u>    | <u>CTCT</u>        | ACAT       | TAAGCACA |          |

Fig. 3 (cont.)

|      | 20    | 30         | 40          | 50         |
|------|-------|------------|-------------|------------|
| 1200 | AGTTT | GGCCTTTATT | CACATTCTTG  | GGGGCGTGAT |
| 1250 | CGGGA | GTATGGCAAT | GAAAGACGGT  | GAGCTGGTGA |
| 1300 | TGTTG | TCTTACACCG | TTTTCATGA   | GCAAACTGAA |
| 1350 | CGCTG | GAATACCAC  | GACGATTTCC  | GGCAGTTTTC |
| 1400 | TGGCA | TGGCGTGTTA | GGGTGAAAAC  | CTGGCTTA   |
| 1450 | GTTTA | TATATGTTTT | TGGTCTCAGC  | CAATCCGTTC |
| 1500 | CCAGT | TTAAACGTG  | CCCAATATGG  | ACAACTTC   |
| 1550 | TTCAC | CAAAATATTA | TACGCAAGGC  | GACAAGGTGC |
| 1600 | GGCGA | TTTCATCATG | CCGTCTGTGA  | TGGCTTCCAT |
| 1650 | TGCTT | ATTACAACAG | TACTGCCGATG | AGTGGCAGG  |
| 1700 | TTTTT | AGATTATTG  | GTGCCCTTAA  | ACGCCTGGGC |
| 1750 | CTAGC | GCATCAAATA | AAACGAAAGG  | CTCAGTCCA  |
| 1800 | TTTCC | TGTGTTGTTT | GTCGGTGAAC  | GCTCTCCTG  |
| 1850 | TCCCG | TAGAGC     |             |            |

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Fig. 4

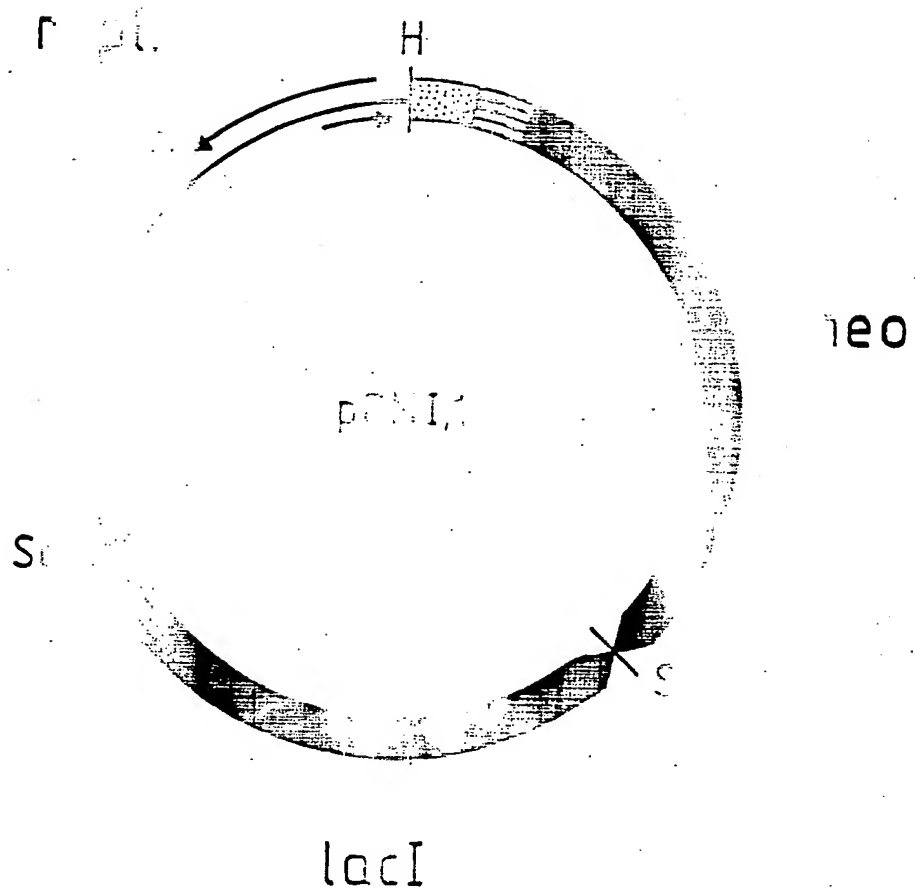


Fig. 5

|   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     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| 185 | 186 | 187 | 188 | 189 | 190 | 191 | 192 | 193 | 194 | 195 | 196 | 197 | 198 | 199 | 200 | 201 | 202 | 203 | 204 | 205 | 206 | 207 | 208 | 209 | 210 | 211 | 212 | 213 | 214 | 215 | 216 | 217 | 218 | 219 | 220 | 221 | 222 | 223 | 224 | 225 | 226 | 227 | 228 | 229 | 230 | 231 | 232 | 233 | 234 | 235 | 236 | 237 | 238 | 239 | 240 | 241 | 242 | 243 | 244 | 245 | 246 | 247 | 248 | 249 | 250 | 251 | 252 | 253 | 254 | 255 | 256 | 257 | 258 | 259 | 260 | 261 | 262 | 263 | 264 | 265 | 266 | 267 | 268 | 269 | 270 | 271 | 272 | 273 | 274 | 275 | 276 | 277 | 278 | 279 | 280 | 281 | 282 | 283 | 284 | 285 | 286 | 287 | 288 | 289 | 290 | 291 | 292 | 293 | 294 | 295 | 296 | 297 | 298 | 299 | 300 | 301 | 302 | 303 | 304 | 305 | 306 | 307 | 308 | 309 | 310 | 311 | 312 | 313 | 314 | 315 | 316 | 317 | 318 | 319 | 320 | 321 | 322 | 323 | 324 | 325 | 326 | 327 | 328 | 329 | 330 | 331 | 332 | 333 | 334 | 335 | 336 | 337 | 338 | 339 | 340 | 341 | 342 | 343 | 344 | 345 | 346 | 347 | 348 | 349 | 350 | 351 | 352 | 353 | 354 | 355 | 356 | 357 | 358 | 359 | 360 | 361 | 362 | 363 | 364 | 365 | 366 | 367 | 368 | 369 | 370 | 371 | 372 | 373 | 374 | 375 | 376 | 377 | 378 | 379 | 380 | 381 | 382 | 383 | 384 | 385 | 386 | 387 | 388 | 389 | 390 | 391 | 392 | 393 | 394 | 395 | 396 | 397 | 398 | 399 | 400 | 401 | 402 | 403 | 404 | 405 | 406 | 407 | 408 | 409 | 410 | 411 | 412 | 413 | 414 | 415 | 416 | 417 | 418 | 419 | 420 | 421 | 422 | 423 | 424 | 425 | 426 | 427 | 428 | 429 | 430 | 431 | 432 | 433 | 434 | 435 | 436 | 437 | 438 | 439 | 440 | 441 | 442 | 443 | 444 | 445 | 446 | 447 | 448 | 449 | 450 | 451 | 452 | 453 | 454 | 455 | 456 | 457 | 458 | 459 | 460 | 461 | 462 | 463 | 464 | 465 | 466 | 467 | 468 | 469 | 470 | 471 | 472 | 473 | 474 | 475 | 476 | 477 | 478 | 479 | 480 | 481 | 482 | 483 | 484 | 485 | 486 | 487 | 488 | 489 | 490 | 491 | 492 | 493 | 494 | 495 | 496 | 497 | 498 | 499 | 500 | 501 | 502 | 503 | 504 | 505 | 506 | 507 | 508 | 509 | 510 | 511 | 512 | 513 | 514 | 515 | 516 | 517 | 518 | 519 | 520 | 521 | 522 | 523 | 524 | 525 | 526 | 527 | 528 | 529 | 530 | 531 | 532 | 533 | 534 | 535 | 536 | 537 | 538 | 539 | 540 | 541 | 542 | 543 | 544 | 545 | 546 | 547 | 548 | 549 | 550 | 551 | 552 | 553 | 554 | 555 | 556 | 557 | 558 | 559 | 560 | 561 | 562 | 563 | 564 | 565 | 566 | 567 | 568 | 569 | 570 | 571 | 572 | 573 | 574 | 575 | 576 | 577 | 578 | 579 | 580 | 581 | 582 | 583 | 584 | 585 | 586 | 587 | 588 | 589 | 590 | 591 | 592 | 593 | 594 | 595 | 596 | 597 | 598 | 599 | 600 | 601 | 602 | 603 | 604 | 605 | 606 | 607 | 608 | 609 | 610 | 611 | 612 | 613 | 614 | 615 | 616 | 617 | 618 | 619 | 620 | 621 | 622 | 623 | 624 | 625 | 626 | 627 | 628 | 629 | 630 | 631 | 632 | 633 | 634 | 635 | 636 | 637 | 638 | 639 | 640 | 641 | 642 | 643 | 644 | 645 | 646 | 647 | 648 | 649 | 650 | 651 | 652 | 653 | 654 | 655 | 656 | 657 | 658 | 659 | 660 | 661 | 662 | 663 | 664 | 665 | 666 | 667 | 668 | 669 | 670 | 671 | 672 | 673 | 674 | 675 | 676 | 677 | 678 | 679 | 680 | 681 | 682 | 683 | 684 | 685 | 686 | 687 | 688 | 689 | 690 | 691 | 692 | 693 | 694 | 695 | 696 | 697 | 698 | 699 | 700 | 701 | 702 | 703 | 704 | 705 | 706 | 707 | 708 | 709 | 710 | 711 | 712 | 713 | 714 | 715 | 716 | 717 | 718 | 719 | 720 | 721 | 722 | 723 | 724 | 725 | 726 | 727 | 728 | 729 | 730 | 731 | 732 | 733 | 734 | 735 | 736 | 737 | 738 | 739 | 740 | 741 | 742 | 743 | 744 | 745 | 746 | 747 | 748 | 749 | 750 | 751 | 752 | 753 | 754 | 755 | 756 | 757 | 758 | 759 | 760 | 761 | 762 | 763 | 764 | 765 | 766 | 767 | 768 | 769 | 770 | 771 | 772 | 773 | 774 | 775 | 776 | 777 | 778 | 779 | 780 | 781 | 782 | 783 | 784 | 785 | 786 | 787 | 788 | 789 | 790 | 791 | 792 | 793 | 794 | 795 | 796 | 797 | 798 | 799 | 800 | 801 | 802 | 803 | 804 | 805 | 806 | 807 | 808 | 809 | 810 | 811 | 812 | 813 | 814 | 815 | 816 | 817 | 818 | 819 | 820 | 821 | 822 | 823 | 824 | 825 | 826 | 827 | 828 | 829 | 830 | 831 | 832 | 833 | 834 | 835 | 836 | 837 | 838 | 839 | 840 | 841 | 842 | 843 | 844 | 845 | 846 | 847 | 848 | 849 | 850 | 851 | 852 | 853 | 854 | 855 | 856 | 857 | 858 | 859 | 860 | 861 | 862 | 863 | 864 | 865 | 866 | 867 | 868 | 869 | 870 | 871 | 872 | 873 | 874 | 875 | 876 | 877 | 878 | 879 | 880 | 881 | 882 | 883 | 884 | 885 | 886 | 887 | 888 | 889 | 890 | 891 | 892 | 893 | 894 | 895 | 896 | 897 | 898 | 899 | 900 | 901 | 902 | 903 | 904 | 905 | 906 | 907 | 908 | 909 | 910 | 911 | 912 | 913 | 914 | 915 | 916 | 917 | 918 | 919 | 920 | 921 | 922 | 923 | 924 | 925 | 926 | 927 | 928 | 929 | 930 | 931 | 932 | 933 | 934 | 935 | 936 | 937 | 938 | 939 | 940 | 941 | 942 | 943 | 944 | 945 | 946 | 947 | 948 | 949 | 950 | 951 | 952 | 953 | 954 | 955 | 956 | 957 | 958 | 959 | 960 | 961 | 962 | 963 | 964 | 965 | 966 | 967 | 968 | 969 | 970 | 971 | 972 | 973 | 974 | 975 | 976 | 977 | 978 | 979 | 980 | 981 | 982 | 983 | 984 | 985 | 986 | 987 | 988 | 989 | 990 | 991 | 992 | 993 | 994 | 995 | 996 | 997 | 998 | 999 | 1000 | 1001 | 1002 | 1003 | 1004 | 1005 | 1006 | 1007 | 1008 | 1009 | 1010 | 1011 | 1012 | 1013 | 1014 | 1015 | 1016 | 1017 | 1018 | 1019 | 1020 | 1021 | 1022 | 1023 | 1024 | 1025 | 1026 | 1027 | 1028 | 1029 | 1030 | 1031 | 1032 | 1033 | 1034 | 1035 | 1036 | 1037 | 1038 | 1039 | 1040 | 1041 | 1042 | 1043 | 1044 | 1045 | 1046 | 1047 | 1048 | 1049 | 1050 | 1051 | 1052 | 1053 | 1054 | 1055 | 1056 | 1057 | 1058 | 1059 | 1060 | 1061 | 1062 | 1063 | 1064 | 1065 | 1066 | 1067 | 1068 | 1069 | 1070 | 1071 | 1072 | 1073 | 1074 | 1075 | 1076 | 1077 | 1078 | 1079 | 1080 | 1081 | 1082 | 1083 | 1084 | 1085 | 1086 | 1087 | 1088 | 1089 | 1090 | 1091 | 1092 | 1093 | 1094 | 1095 | 1096 | 1097 | 1098 | 1099 | 1100 | 1101 | 1102 | 1103 | 1104 | 1105 | 1106 | 1107 | 1108 | 1109 | 1110 | 1111 | 1112 | 1113 | 1114 | 1115 | 1116 | 1117 | 1118 | 1119 | 1120 | 1121 | 1122 | 1123 | 1124 | 1125 | 1126 | 1127 | 1128 | 1129 | 1130 | 1131 | 1132 | 1133 | 1134 | 1135 | 1136 | 1137 | 1138 | 1139 | 1140 | 1141 | 1142 | 1143 | 1144 | 1145 | 1146 | 1147 | 1148 | 1149 | 1150 | 1151 | 1152 | 1153 | 1154 | 1155 | 1156 | 1157 | 1158 | 1159 | 1160 | 1161 | 1162 | 1163 | 1164 | 1165 | 1166 | 1167 | 1168 | 1169 | 1170 | 1171 | 1172 | 1173 | 1174 | 1175 | 1176 | 1177 | 1178 | 1179 | 1180 | 1181 | 1182 | 1183 | 1184 | 1185 | 1186 | 1187 | 1188 | 1189 | 1190 | 1191 | 1192 | 1193 | 1194 | 1195 | 1196 | 1197 | 1198 | 1199 | 1200 | 1201 | 1202 | 1203 | 1204 | 1205 | 1206 | 1207 | 1208 | 1209 | 1210 | 1211 | 1212 | 1213 | 1214 | 1215 | 1216 | 1217 | 1218 | 1219 | 1220 | 1221 | 1222 | 1223 | 1224 | 1225 | 1226 | 1227 | 1228 | 1229 | 1230 | 1231 | 1232 | 1233 | 1234 | 1235 | 1236 | 1237 | 1238 | 1239 | 1240 | 1241 | 1242 | 1243 | 1244 | 1245 | 1246 | 1247 | 1248 | 1249 | 1250 | 1251 | 1252 | 1253 | 1254 | 1255 | 1256 | 1257 | 1258 | 1259 | 1260 | 1261 | 1262 | 1263 | 1264 | 1265 | 1266 | 1267 | 1268 | 1269 | 1270 | 1271 | 1272 | 1273 | 1274 | 1275 | 1276 | 1277 | 1278 | 1279 | 1280 | 1281 | 1282 | 1283 | 1284 | 1285 | 1286 | 1287 | 1288 | 1289 | 1290 | 1291 | 1292 | 1293 | 1294 | 1295 | 1296 | 1297 | 1298 | 1299 | 1300 | 1301 | 1302 | 1303 | 1304 | 1305 | 1306 | 1307 | 1308 | 1309 | 1310 | 1311 | 1312 | 1313 | 1314 | 1315 | 1316 | 1317 | 1318 | 1319 | 1320 | 1321 | 1322 | 1323 | 1324 | 1325 | 1326 | 1327 | 1328 | 1329 | 1330 | 1331 | 1332 | 1333 | 1334 | 1335 | 1336 | 1337 | 1338 | 1339 | 1340 | 1341 | 1342 | 1343 | 1344 | 1345 | 1346 | 1347 | 1348 | 1349 | 1350 | 1351 | 1352 | 1353 | 1354 | 1355 | 1356 | 1357 | 1358 | 1359 | 1360 | 1361 | 1362 | 1363 | 1364 | 1365 | 1366 | 1367 | 1368 | 1369 | 1370 | 1371 | 1372 | 1373 | 1374 | 1375 | 1376 | 1377 | 1378 | 1379 | 1380 | 1381 | 1382 | 1383 | 1384 | 1385 | 1386 | 1387 | 1388 | 1389 | 1390 | 1391 | 1392 | 1393 | 1394 | 1395 | 1396 | 1397 | 1398 | 1399 | 1400 | 1401 | 1402 | 1403 | 1404 | 1405 | 1406 | 1407 | 1408 | 1409 | 1410 | 1411 | 1412 | 1413 | 1414 | 1415 | 1416 | 1417 | 1418 | 1419 | 1420 | 1421 | 1422 | 1423 | 1424 | 1425 | 1426 | 1427 | 1428 | 1429 | 1430 | 1431 | 1432 | 1433 | 1434 | 1435 | 1436 | 1437 | 1438 | 1439 | 1440 | 1441 | 1442 | 1443 | 1444 | 1445 | 1446 | 1447 | 1448 | 1449 | 1450 | 1451 | 1452 | 1453 | 1454 | 1455 | 1456 | 1457 | 1458 | 1459 | 1460 | 1461 | 1462 | 1463 | 1464 | 1465 | 1466 | 1467 | 1468 | 1469 | 1470 | 1471 | 1472 | 1473 | 1474 | 1475 | 1476 | 1477 | 1478 | 1479 | 1480 | 1481 | 1482 | 1483 | 1484 | 1485 | 1486 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---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Fig. 5(cont.)

|      | 10  | 20          | 30         | 50         |
|------|-----|-------------|------------|------------|
| 1900 | TCA | TTCAGGCATTT | GCATGGTTTG | TTGAAA     |
| 1950 | CCC | TTCCCGTTCC  | GCTATCGGCT | GAATTT     |
| 2000 | CCC | AGCCAGCCAG  | ACCCAGAGCC | GCCGAC     |
| 2050 | AAC | AGCGCGATTT  | GCTGGTCCC  | CAATCA     |
| 2100 | TCG | CGTACCGTCT  | TCATGGGAGA | AAATAA     |
| 2150 | CAG | AGACATCAAG  | AAATAAACC  | GGAACA     |
| 2200 | GCA | ATGGCATCGT  | GCTCATCCAG | CGGATA     |
| 2250 | GGG | TTGGCGGAGA  | AGATTGTCCA | CGGCGG     |
| 2300 | ATC | GTTCTACCAT  | CGCAGCACC  | AGGCTA     |
| 2350 | TAT | TTAATCGGCG  | CGACAATTTC | CGACCT     |
| 2400 | AGT | GGCAACGCCA  | ATTAGCAGG  | AGTGCT     |
| 2450 | TCG | GCTTGGGAAT  | GCATTC     | GC TCCGCT  |
| 2500 | CGC | GTTTTCCGAG  | AA GGT GGT | GCCCTT     |
| 2550 | ATG | ATAGGAGAT   | CG TCA GCT | GTGGG      |
| 2600 | ACA | CATTCACCC   | GCTCAATTC  | CTCTCT     |
| 2650 | ACG | CGAAAGGTTT  | TCCAGC     | ATC CATGCT |
| 2700 | ATC | GGCTTCGGCG  | GCAATCTC   | CAACCT     |
| 2750 | ACG | GGCTCGTGGG  | TAGGGGAAA  | AGGAGC     |
| 2800 | AGT | GTATACTCGG  | TTACTATCT  | GCGCAT     |
| 2850 | ATT | CATGTGGGAG  | GA TAA     | AAG GGTCT  |
| 2900 | TGA | TACAGGATAT  | AT TCG     | ATC CTGGC  |
| 2950 | CGT | TGGCTGCT    | CT TCG     | AAA TGGCT  |
| 3000 | GGG | AAGATGCCCG  | GAGGATAGTT | AAGAGC     |
| 3050 | AGC | GCTTTTTCGA  | TAGGCTGGG  | CGGCGA     |
| 3100 | AGC | TCAATCTCT   | GCTGGC     | AAA GCGCA  |
| 3150 | ATT | TGGCTGCTCG  | GCTGGC     | GCT GGGC   |
| 3200 | CAC | GGGTGTCAAT  | CGCTG      | TAT GGGC   |
| 3250 | ACA | CTGCTTCTCG  | GCAGG      | AGT TGGC   |
| 3300 | AGC | GGGCTTCTCT  | TC TAC     | GCT GGGCG  |
| 3350 | AGT | CGAAGCCGCA  | A TACA     | CGA AAAGC  |
| 3400 | ATT | TGATTTACCG  | GCTTACTCT  | TGAAG      |
| 3450 | TC  | AAAGGAGAG   | TATGCGGAT  | TGGCT      |
| 3500 | ATC | AAAAGCTGCG  | TATTTT     | CAG AACCT  |
| 3550 | ATT | TTT TCG     | AT TCAAG   | AAA ACAT   |
| 3600 | AGG | AAGTCAATCT  | TAT TAC    | CAG ATAAA  |
| 3650 | ATT | ATGCTTCAAA  | AT TAA     | AGC TGAAG  |
| 3700 | ATT | ATGCTTCAAA  | TAT TAA    | GCT TATCA  |

Fig. 6

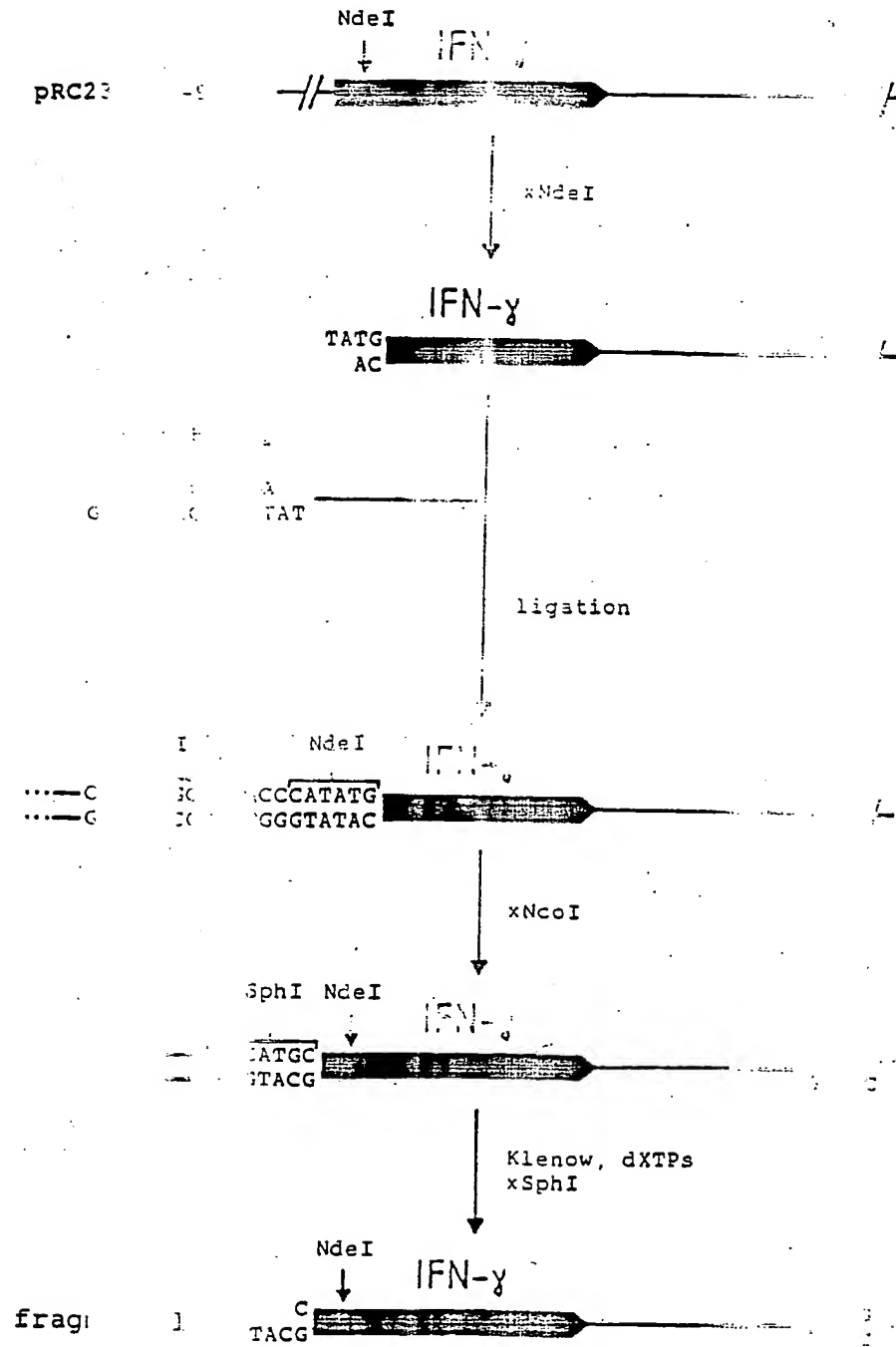


Fig. 7

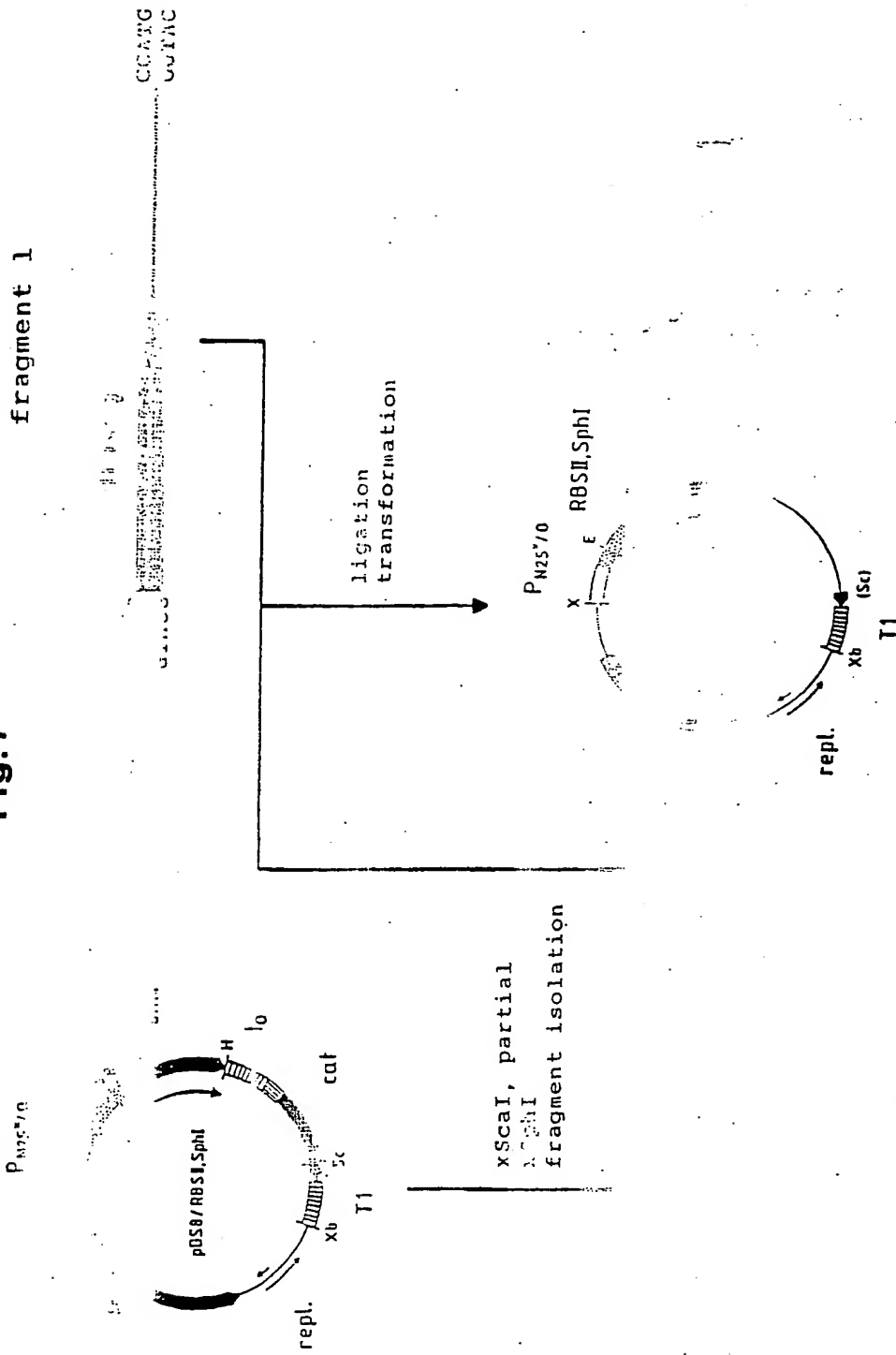


Fig. 8

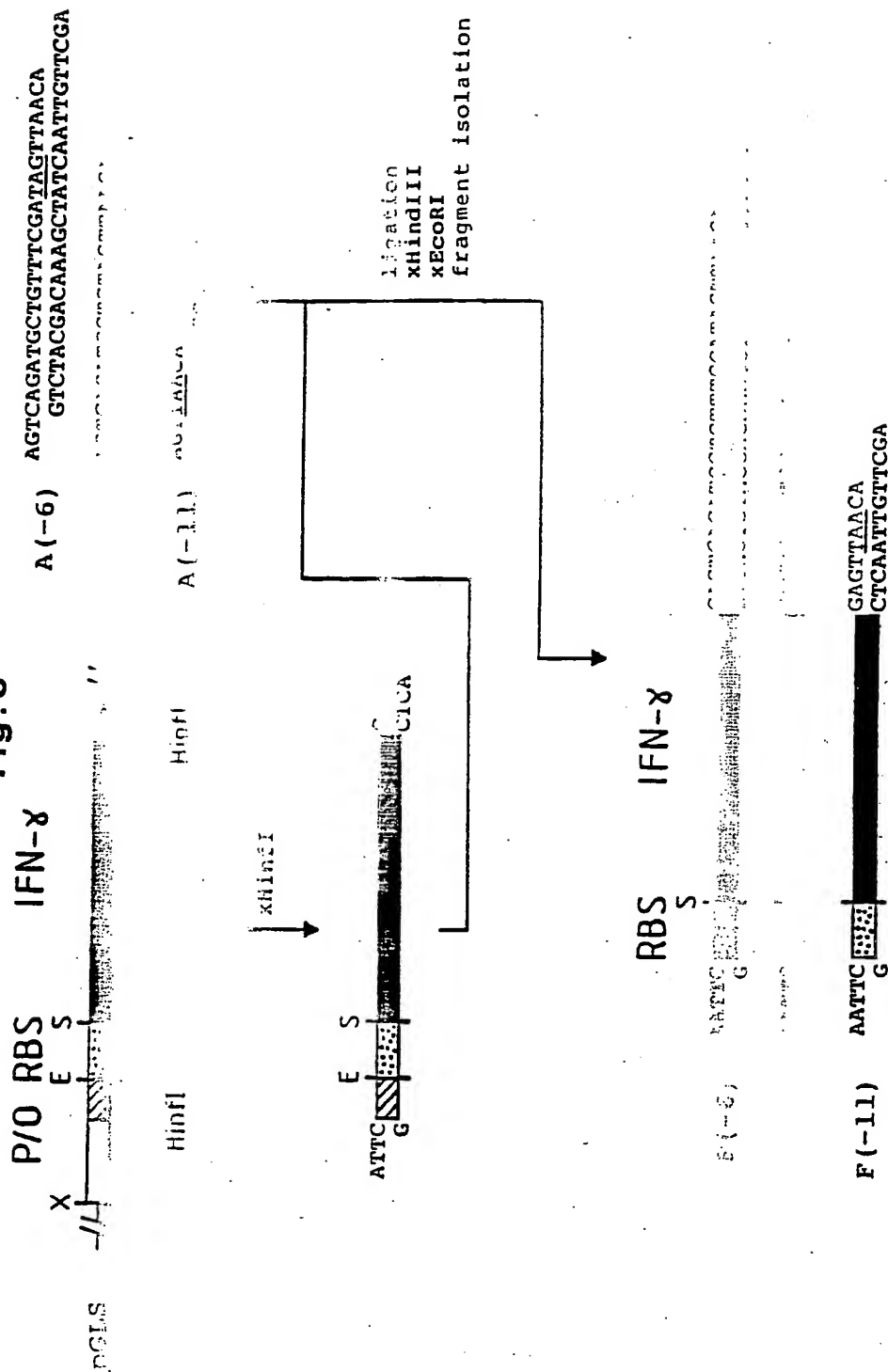


Fig.9

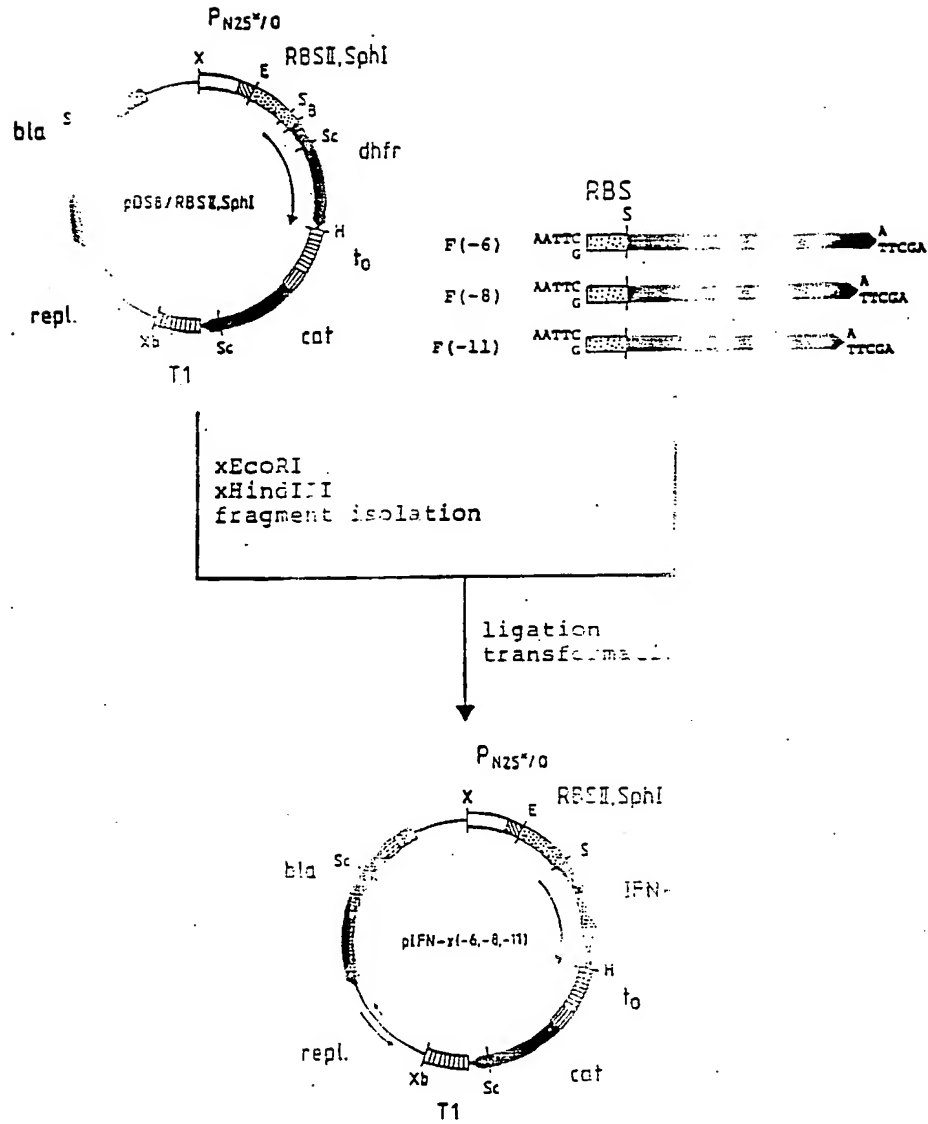
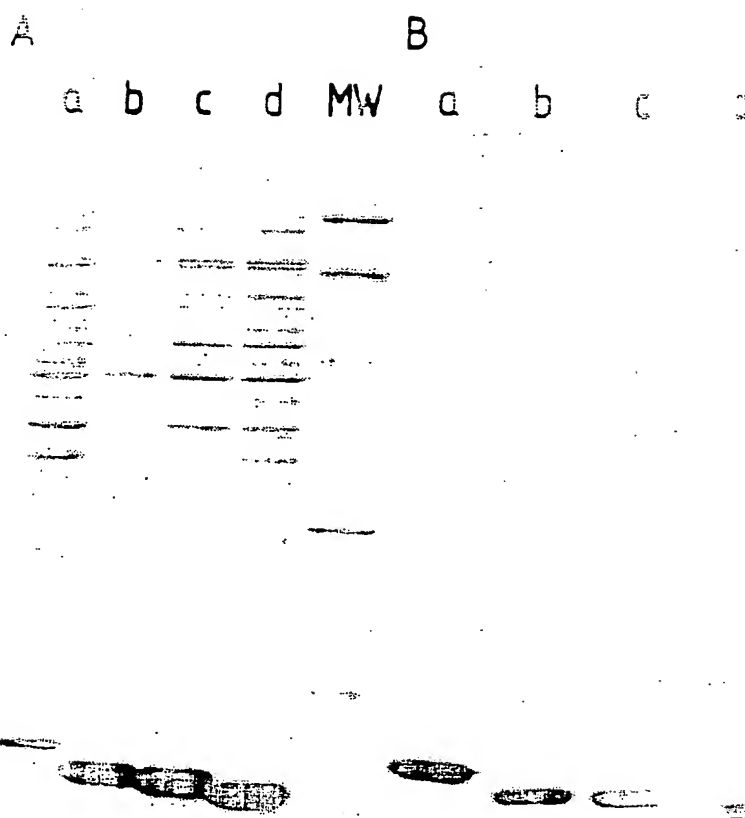


Fig. 10

IFN- $\gamma$ (-6) Met-(130aa)-ArgSerGlnMetLysPhe  
 IFN- $\gamma$ (-7) Met-(130aa)-ArgSerGlnMetLysPhe  
 IFN- $\gamma$ (-8) Met-(130aa)-ArgSerGlnMetLys  
 IFN- $\gamma$ (-9) Met-(130aa)-ArgSerGlnMet  
 IFN- $\gamma$ (-10) Met-(130aa)-ArgSerGln  
 IFN- $\gamma$ (-11) Met-(130aa)-ArgSer

Fig.11a



1b

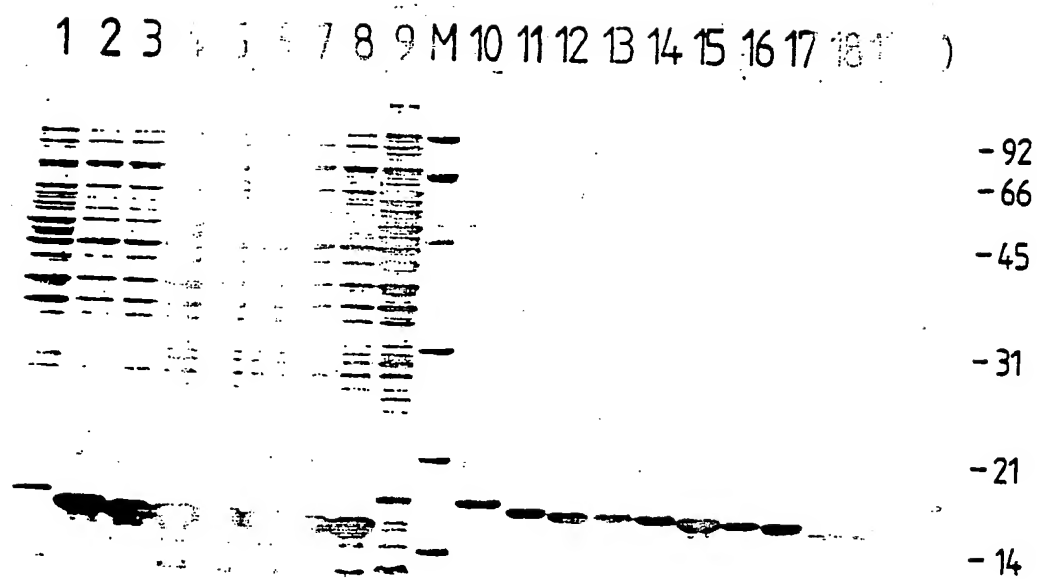




Fig.12

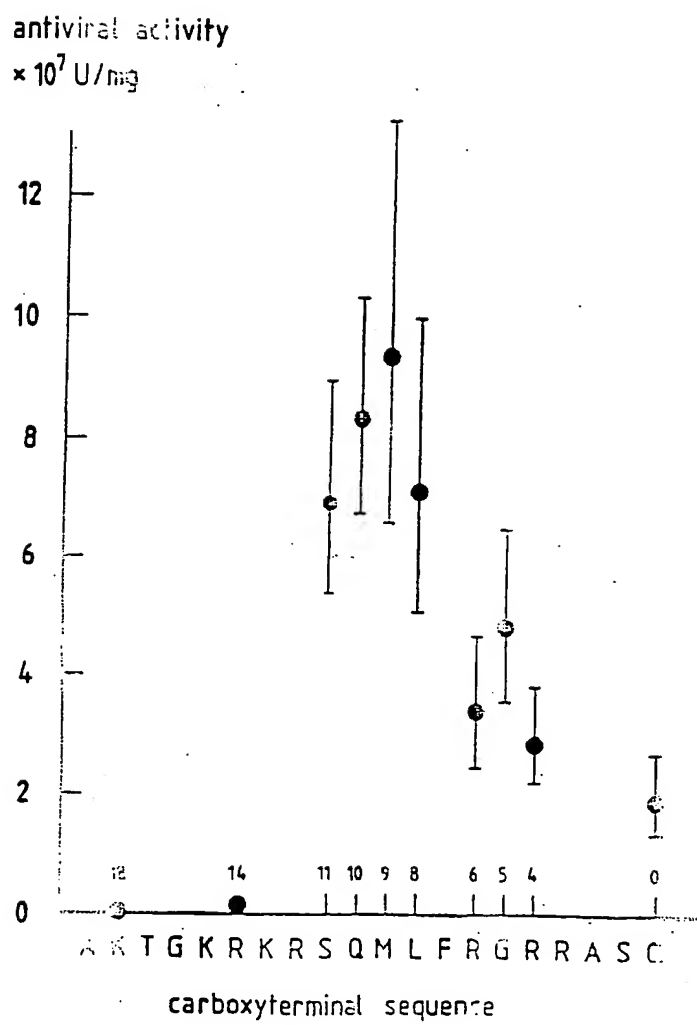
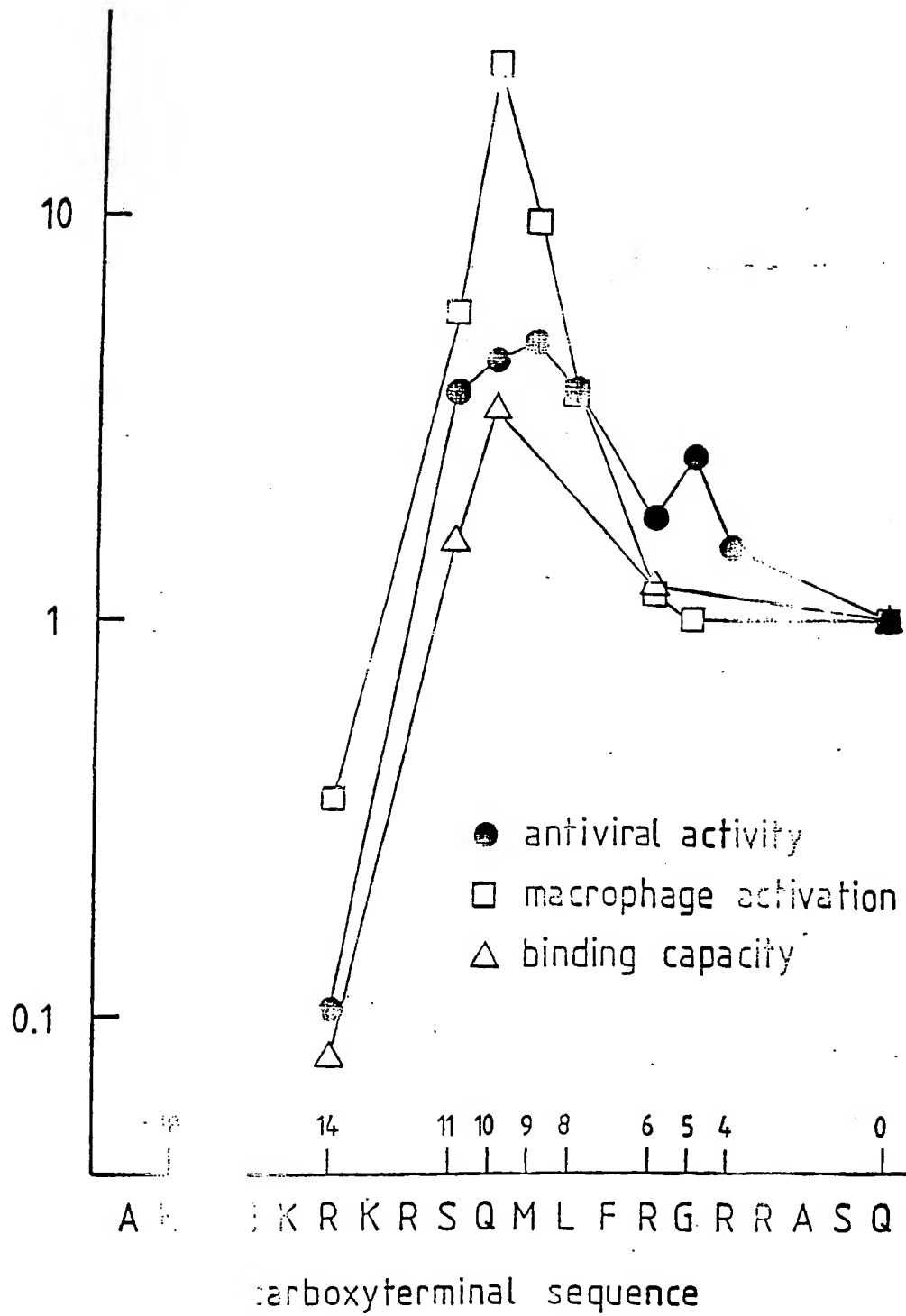


Fig. 13



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